Structural Investigation of the Water-Soluble Polysaccharides of *Cladophora rupestris*.

Thesis for the Degree of Doctor of Philosophy

by

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CONTENTS.

INTRODUCTION.

G E N E R A L.

Seaweeds belong to the large group of plants known as the Algae. This group, which evolved very early in the botanical history of the world, contains some of the most primitive members of the plant kingdom and many of these have not altered vitally from their ancestral forms. The seaweeds, about 17,000 species of which have been described, are conveniently sub-divided into four main classes according to the nature and proportions of their colouring pigments. These are the green (Chlorophyceae), brown (Phaeophyceae), red (Rhodophyceae) and blue-green (Myxophyceae) seaweeds. Like land plants they require sunlight, carbon-dioxide and minerals for growth and their principal organic components are carbohydrates, proteins, fats, pigments, sterols and vitamins. Unlike land plants, however, they possess no roots, differ in their mode of reproduction and are subject to wide variations in composition according to changes in season and environment.

From pre-Christian times references exist to the utilisation of marine algae. Japan and China, for instance, possess flourishing seaweed industries of great antiquity and a large part of the annual harvest is devoted to human consumption. In general the food value of these plants is low and their main contribution to diet is as roughage and in the provision of vitamins and minerals; indeed, the low

incidence of goitre in Japan is attributed to the iodine content of the edible seaweeds. In the Western World the whole weed has never enjoyed wide popularity as a food, although in earlier times certain species, for example Rhodymenia palmata, were eaten in Scotland, Ireland and Scandinavia. Even today, the red weed, Porphyra, or "laver" and the green weed Ulva lactuca or "sea lettuce", are still culinary dishes in certain parts of South Wales, Devon and Cornwall. Although lacking in popular appeal, seaweeds do contribute indirectly to human nutrition through their use as fertilisers and soil improvers and through their contribution to animal feeding stuff.

In the 18th and 19th centuries the extraction of iodine, potash and soda from seaweed ash was a vigorous though disorganised industry in the Scottish Highlands and Islands, in Ireland and also in French coastal regions, where it originated. This industry has now largely died out but dwindling mineral deposits of iodine may one day necessitate its revival. Vigorous research over the last few decades has, however, led to many new applications for seaweed products. At the present time only the carbohydrate extracts from the brown and red algae are commercially important and these provide raw materials for the chemical, pharmaceutical, textile, rubber, paint and paper industries, as well as for the food and fertiliser industries.

The main polysaccharide extracted from brown seaweeds, alginic acid, was first manufactured in Britain in 1910 and,

since that time, a flourishing alginate industry has developed in this country, second only to that of America. These alginate salts function mainly as thickening, emulsifying stabilising and gelling agents in many of the industries already mentioned. The other principal extracts of the brown weeds are the sugar-alcohol, mannitol, the 1:3 -β- glucosan, laminarin, and the sulphated fucosan, fucoidin. Of these, mannitol and, to a lesser extent, laminarin, show promise of commercial importance. Mannitol, at present manufactured synthetically from glucose, has already found applications in the explosives, paints, electrical and pharmaceutical industries and laminarin has been used as a soluble surgical dusting powder, while its partly sulphated derivative may prove to be an effective blood anti-clotting agent.

The polysaccharide agar is derived from Gelidium, Gracilaria and Euchema species of the red seaweeds. Before the second world war Japan was its principal producer maintaining an industry which originated in the 17th century, but in more recent years other countries have embarked on its manufacture. The most important use for agar is as a biological medium in the preparation of vaccines and in bacterial and fungal cultures, since it possesses the dual properties of high gelling strength over a wide temperature range and resistance to liquefaction by bacterial cultures. Indeed it was these uses for agar which made it an essential commodity in the second world war and which led British

manufacturers to seek a substitute in carrageenin, an extract of the red weeds Chondrus crispus and Gigartina stellata. Carrageenin or "British Agar" is claimed to be superior to the Japanese product in the matter of clarity, lower melting point and solubility.

Agar is also widely employed as an emulsifying, stabilising and thickening agent in the food industries and as a preservative in the canning industry. It serves as a base in shoe stains, as a waterproofing and soundproofing agent and as a clarifier in the manufacture of beers, wines and spirits.

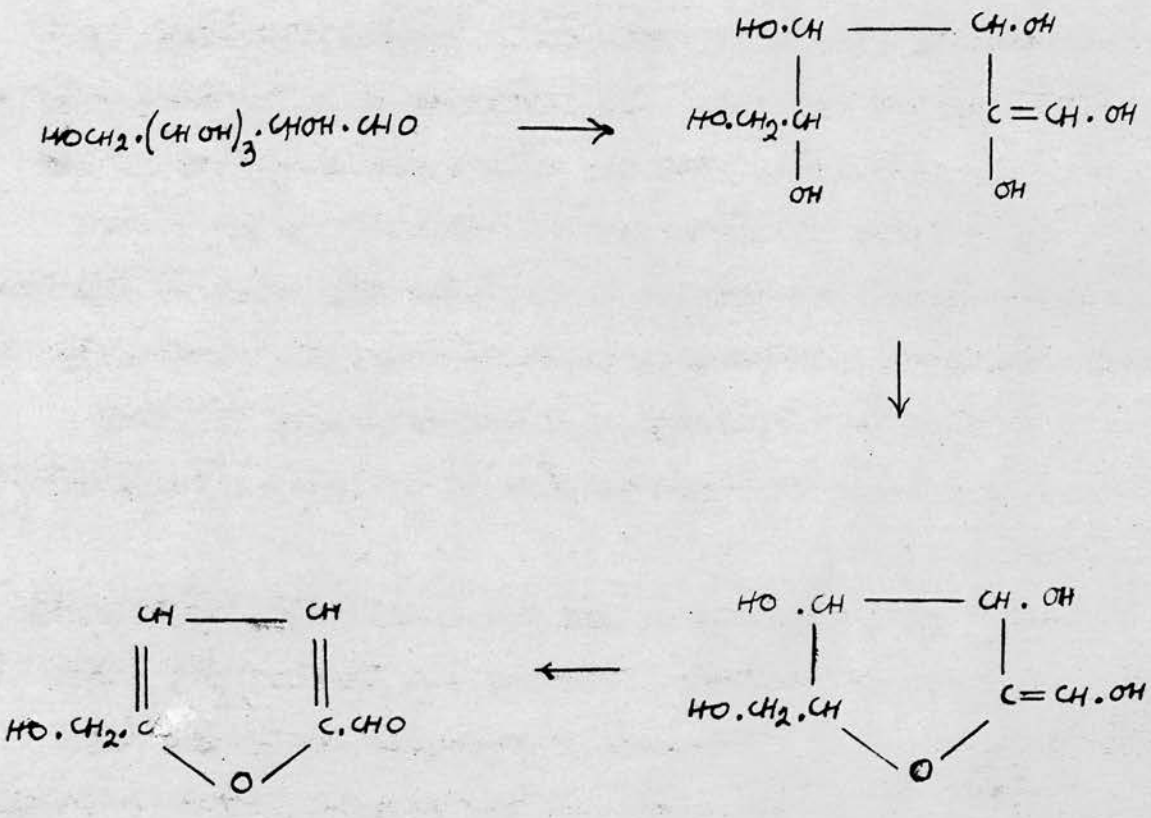
Very little is at present known about the chemistry of the Chlorophyceae and Myxophyceae and very little commercial value is attached to them. However, in recent years, increasing attention has been paid to the unicellular fresh water algae such as Chlorella, often observed as a green scum floating on inland ponds. Apart from their rapidity of growth these algae have the advantage over land plants in that they possess protein contents of up to 88% of the dry plant (1). Intensive investigations into the mass culture of such organisms are now in progress and, depending on the success of these experiments, it is possible that factory produced microscopic algae may be used as food in the protein deficient countries of the East. At the present time, however, only the alginate and agar industries can truly be regarded as flourishing. Mainly owing to the difficulties of large scale mechanical harvesting and processing, other algal products cannot be

manufactured at competitive prices. Nevertheless, a growing awareness of the great abundance and variety of the seaweeds will no doubt continue to stimulate efforts towards their further industrial utilisation.

The Polysaccharide Sulphates of Marine Algae.

Many seaweed polysaccharides are characterised by the presence of sulphate residues which were first reported by Haas in carrageenin (2). These incorporate the basic repeating unit $R.O.SO_2.OM$ where R represents the sugar residue and M is a cation. In no case has complete clarification of the structure of these polysaccharides been achieved owing to the complexity of the molecules and to the presence of the chemically resistant sulphate grouping.

Carrageenin is obtained by aqueous extraction of the red weed, Chondrus crispus, and polysaccharides of similar composition and properties are obtained from the closely related weeds Gigartina stellata (3) and Chondrus ocellatus (4). The extracts contain about 30% of organically bound sulphate, the cations being mainly sodium and potassium in the cold water extracts and calcium in the hot water extracts of the weed (5). The main organic component, D-galactose, was first reported by Fluckiger and Obermaier in 1868 (6) and this sugar has subsequently been shown to comprise about two thirds of the carbohydrate constituents of the polysaccharide (5). Small quantities of D-glucose and pentose material were found by Sebor (7) and later the presence of L-galactose was established (8). The principal remaining constituent 3:6-anhydro-D-galactose has recently been reported independently by E.E. Percival (9) and by O'Neill (10). The presence of a ketose has been reported by various workers (3, 5, 6, 7, 11, 12) but no such compound

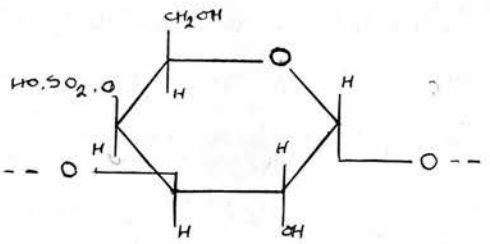


5 - hydroxymethyl - 2 - furaldehyde. (I).

has ever been isolated. Young and Rice (13) have described the isolation of the di-O-isopropylidine derivative of 2-keto-D-gluconic acid in 3% yield but other investigators (3, 5) were unable to repeat the work and this compound is generally regarded as an artefact. The ketose reactions are now ascribed to 5-hydroxymethyl-2-furfuraldehyde, the acid degradation product of 3:6-anhydro-D-galactose (14). This latter molecule, incorporating both a pyranose and a 3:6-anhydro ring, is a strained system and since the formation of a furanose ring is sterically prevented, probably exists predominantly in the open-chain form. This structure, which contains a free aldehydic group is regarded as the starting point of the breakdown reactions, 1:2-enediol probably being the first stage in the breakdown to 5-hydroxymethyl-2-furaldehyde (I) (see Haworth and Jones (15) and Wolfrom et al (16) on the breakdown of glucose.)

2-O-Methyl-D-galactose and 2:6-di-O-methyl-D-galactose were isolated by Buchanan, Percival and Percival (5) from the hydrolysate of methylated carrageenin indicating that positions 3 and 4 were available for esterification by sulphate radicals or for union with adjacent galactose residues. Owing to the ease with which monosaccharide - 6 - and - 3 - sulphates are degraded by alkali to 3:6 - anhydro derivatives (17, 18, 19) whenever this is sterically feasible, and to the resistance to alkaline hydrolysis evidenced by the carrageenin sulphate (20), C_4 was proposed as the most probable location

for this group. The main repeating unit put forward was:



The pyranose configuration was favoured in view of the slow rate of hydrolysis as compared with carulose, a poly-galactofuranose (21). The α - linkage was based on the positive rotation of the polysaccharide and the fall in value of this rotation on hydrolysis.

Further support for this structure was obtained by Johnston and Percival (8) through the isolation of 2:4:6-tri-O-methyl-D-galactose, 2:3:4:6-tetra-O-methyl-D-galactose and 2:6-di-O-methylgalactose from the hydrolysate of partially degraded methylated carrageenin. By treatment with cold methanolic hydrogen chloride these workers also isolated a resistant desulphated fragment in 15% yield (8) which gave rise, on methylation followed by hydrolysis, to tetra-O-methyl-D-galactopyranose (1 part), 2:4:6-tri-O-methyl-D-galactose (2 parts), 2:4-di-O-methyl-D-galactose (4 parts), tetra-O-methyl-L-galactopyranose (2 parts) and 2:4:6-tri-O-methyl-L-galactose (6 parts). The high proportion of tetramethyl and of dimethyl sugars led these authors to suggest a branched

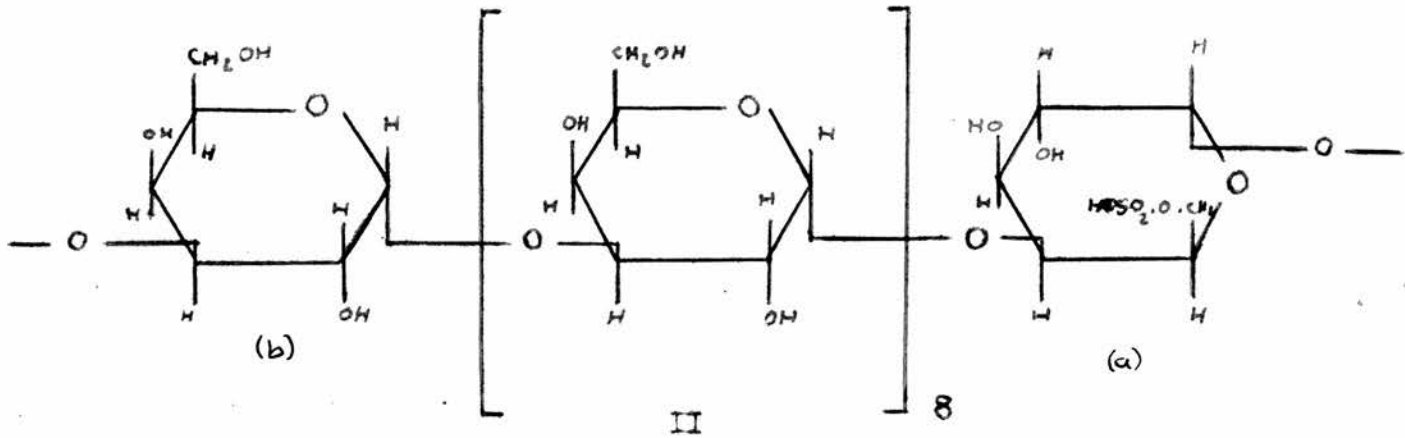
structure involving C_1 to C_6 linkages. Independent supporting evidence for this was furnished by Dillon and O'Colla (22) who isolated 2:4:6-tri-O-methyl-D-galactose and 2:4-di-O-methyl-D-galactose, after methylation and hydrolysis of a sulphur-free, acetylated and heterogeneously degraded carrageenin.

The homogeneous nature of carrageenin has long been contested (2, 8, 12, 23 - 26) but it is only recently that ultra-centrifuge experiments have revealed the presence of two components (26) and a new method for their macro-separation has been devised, based on the gelatinising effect of the potassium ion (27, 28). Sharp fractionation occurs on the addition of solid potassium chloride to dilute solutions of the sodium salt. This results in the precipitation of a potassium sensitive fraction (40%), designated κ - carrageenin, leaving in solution λ - carrageenin (60%). Smith, O'Neill and Perlin (29) have found κ - carrageenin to consist of approximately equal amounts of D-galactose and 3:6-anhydro-D-galactose with 25% of combined sulphate. This fraction is not susceptible to periodate oxidation and thus has no free adjacent hydroxyl groups in its sugar units. This fact is consistent with the formulation of κ - carrageenin as a chain of D-galactose-4-sulphate residues glycosidically linked through C_1 and C_3 and involving 3:6-anhydro-D-galactose units. The structure could be either linear or branched but in the latter case the branches must be terminated by 3:6-anhydrogalactose residues since end units of D-galactose-4-sulphate residues

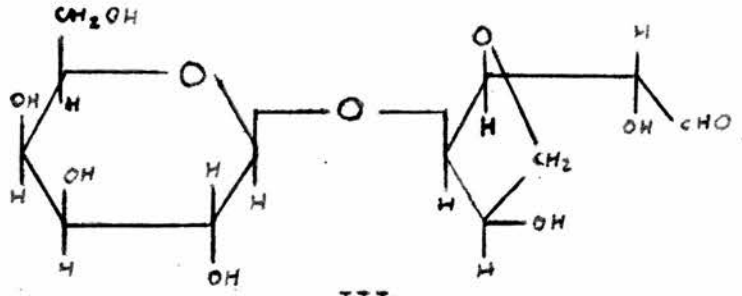
would make the κ - carrageenin vulnerable to attack by periodate at the 2:3-glycol position. By mercaptolysis of κ - carrageenin, O'Neill (30) has recently reported the isolation from the mother liquor of the derivative of a disaccharide whose structure he characterised as 4-O-β-D-galactopyranosyl - 3:6-anhydro-D-galactose.

Graded alcohol fractionation of λ - carrageenin separated a main fraction (62%) containing D-galactose with 35% esterified sulphate and a trace of 3:6-anhydro-D-galactose probably present as an impurity. Periodate uptake and formic acid release determinations on this fraction showed a consumption of 0.31 moles of periodate and a release of 0.13 moles of formic acid for every sugar residue. It would appear that one sugar unit in five must have at least one free diol group and one in eight a free triol group. This could not be accommodated by chains of 1:3 linked D-galactose-4-sulphate residues but a branched structure with some residues unesterified could account for the periodate consumption. A second alcohol fraction (11%) consisted of D- and L-galactose with lesser amounts of glucose and xylose and a final fraction (1.4%) contained the last three sugars with only a trace of D-galactose. Since L-galactose was found only in the last two fractions these authors consider that it cannot therefore form part of the main polysaccharide. The glucose and xylose probably arise from associated glucosans and xylans.

Agar, the polysaccharide extracted from Gelidium spp. and related red weeds has a much lower sulphur content (0.3 - 1.5%) (19, 31 - 33) than carrageenin but, like the latter polysaccharide



II



III

is built up from galactose residues. Percival and Somerville (34) isolated 2:4:6-tri-O-methyl-D-galactose (65%) as the major hydrolysis product of methylated agar, together with a mono-O-methyl fraction which was further fully methylated to a di-O-methyl derivative. This was considered to be 3:6-anhydro - 2:4-di-O-methyl-L-galactose (35) and its structure was subsequently confirmed by the synthesis of its enantiomorph by Forbes and Percival (36). Further evidence for the presence of this anhydro derivative was provided by Araki and Arai (37) who isolated 3:6-anhydro-2-O-methyl-L-galactose on acetolysis of the methylated agar from Gelidium amansii.

Jones and Peat (38) considered that the 3:6-anhydride arose through the alkaline hydrolysis of L-galactose-6-sulphate residues during the course of methylation. In view of the above facts and of the proportion of the different methylated derivatives isolated these authors put forward a formula (38) for agar consisting of unit chains of approximately nine D-galactopyranose residues joined by 1:3-β-glycosidic links and terminated at the reducing end by a 1:4 linked L-galacto-pyranose-6-sulphate unit (II). They considered the whole polysaccharide to be built up of aggregates of these chains united through the reducing group of the L-galactose residue (a) and the third carbon atom of the terminal non-reducing D-galactose unit (b).

However, this structure is by no means in agreement with all the experimental evidence. Whereas the highest recorded methoxyl value for methylated agar is 35% (38) a fully

methylated derivative with the above constitution would contain 42% methoxyl. Furthermore, as pointed out by E.G.V. Percival and others (17, 19, 31, 39, 40) the sulphur content of agar is too low to account for the high proportion of anhydro units in its methylated derivative, and Percival considered that such units were already present in the unmethylated agar and that they may have resulted from its desulphation during manufacture or even during the elaboration of the polysaccharide by the alga itself. Subsequent experimental work has confirmed this view.

Araki (41) has shown that the agar molecule contains only 21% of hydroxyl groups available for methylation. He has also found that complete methanolysis gave 3:6-anhydro-L-galactose dimethyl acetal in 17 - 20% yield (42) and partial methanolysis (43) gave 45 - 50% of the dimethyl acetal of a disaccharide "agarobiose" which was shown to be 4-β-D-galactopyranosyl-3:6-anhydro-L-galactopyranose (III).

By the enzymatic hydrolysis of agar, Araki and Arai (44) have recently isolated a new disaccharide termed "neoagarobiose" the structure of which was established as 3-α-D-galactopyranosyl-3:6-anhydro-L-galactopyranose.

In the light of all this evidence it is probable that 3:6-anhydro-L-galactose is a constituent of the agar molecule. However, conclusive evidence for the location of the sulphate group is lacking. Percival has demonstrated that methyl glucofuranoside-3-sulphate and methyl glucofuranoside-6-sulphate

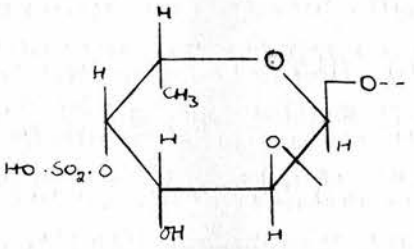
readily yield a mixture of 3:6-anhydroglucose and glucose on alkaline hydrolysis (19) and suggested that the sulphate group in the L-galactose residue may equally well reside on C_3 instead of C_6 as was originally postulated by Jones and Peat (38).

It must be pointed out that, except for sulphur estimations (19), these investigations have not been carried out on agar from pure botanical sources, commercial samples of unspecified history have been used. Small amounts of pentose 1.8 - 5.7% (45 - 47), methyl pentose 1.0 - 3.6% (45, 47 - 49) and uronic acid 3.6 - 7.4% (48,49) have been reported but it is not known whether these are simply adventitious impurities or whether they form an integral part of the main polysaccharide and no account has been taken of them in structural discussions. More evidence, including molecular weight determination, is required before the absolute structure of agar can be established.

Fucoidin, the principal carbohydrate ethereal sulphate of the Phaeophyceae, was first described and named by Kylin (50). Percival and Ross (51) have isolated this polysaccharide from Himanthalia lorea as well as from species of Fucus and Laminaria and subjected the extracts to intensive purification. The highly purified specimens contained 38.3% of combined sulphate, calcium being the principal cation together with a little sodium, potassium and magnesium. L-Fucose constituted about 86% of the organic matter and the small amounts of glucose, uronic acid and xylose also present were regarded as impurities.

The hydrolysates from methylated fucoidin were shown by Conchie and Percival (52) to contain L-fucose (ca. 1 part),

3-O-methyl-L-fucose (ca. 3 parts) and 2:3-di-O-methyl-L-fucose (ca. 1 part). A sulphate group attached to either C_2 or C_3 in L-fucose was shown by Percival (53) to be alkali labile. The resistance of fucoidin to alkaline hydrolysis and the isolation of 3-O-methyl-L-fucose as the major hydrolysis product of methylated fucoidin proves that the predominant glycosidic linkage is C_1 to C_2 and that the sulphate group must be on C_4. The negative rotation of the polysaccharide and its derivatives suggests the α-L-sugar and a principal repeating unit of:



The presence of free fucose in the methylated material led Percival to suggest a branched structure for the polysaccharide but more work is necessary before a unique structure can be assigned to it.

The polysaccharide obtained from the red seaweed Dilsea edulis has been studied by Dillon and his colleagues (54, 55). They found that the free acid contains about 12% of combined sulphate, 70% D-galactose and 9.6 - 11% uronic acid. This extract differs from carrageenin in forming highly viscous,

non-gelling solutions and neither L-galactose nor 3:6-anhydro-hexose residues appear to be present.

Degradative acetylation followed by methylation and hydrolysis gave 2:4:6-tri-O-methyl-D-galactose, 2:3:4:6-tetra-O-methyl-D-galactose, di-O-methyl-D-galactose and a trace of 2:3:6-tri-O-methyl-D-galactose. These results indicate that the molecule is made up of chains of galactose units linked principally C_1 to C_3 with some C_1 to C_4 links, but no C_1 to C_6. The presence of dimethyl galactose may indicate a branched molecule, and periodate oxidation experiments show that only one galactose unit in five has free adjacent hydroxyl groups. The sulphate content corresponds to one ester grouping to every four or five sugar residues and its alkali stability suggests C_4 as a possible location. The uronic acid content is not significantly changed by any purification or degradation reactions but no account has yet been taken of its function in the polysaccharide.

The only sulphated polysaccharide from the Chlorophyceae so far studied is that from Ulva lactuca. Brading, Georg-Plant and Hardy (56) showed that the extract from this seaweed contains D-xylose, L-rhamnose, D-glucose and D-glucuronic acid in the percentage proportions 9.4: 31: 7.7: 19.2. This, incidentally, is the first reported occurrence of rhamnose in an algal polysaccharide. Combined sulphate is present in the free acid to the extent of 15.9% and is stable to alkali. Attempts to separate this material into more than one entity were unsuccessful but after methylation, chloroform soluble and insoluble fractions were obtained. The chloroform soluble

fraction (17% of the total) after further methylation had a methoxyl content of 31%, a sulphate of 3% and gave on hydrolysis 2:3:4-tri-O-methyl-L-rhamnose, 2:3-di-O-methyl-D-glucose and 2:3:4-tri-O-methyl-D-glucose. The chloroform insoluble material, which appeared to be free from glucose, had a methoxyl content of 17.2% and a sulphate content of 30%. On hydrolysis it was shown by paper chromatography to contain 2:3:4-tri-O-methyl-D-xylose and 2:3-di-O-methyl-D-xylose, 2:3:4-tri-O-methylrhamnose and 2:3-di-O-methylrhamnose together with free rhamnose and xylose.

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EXPERIMENTAL SECTION.

General Methods used throughout the Work.

Unless otherwise stated:

Evaporations were carried out at 40° under reduced pressure.

Samples for analysis were dried overnight in vacuo over phosphorus pentoxide.

Nitrogen contents (N) were estimated by a semi-micro-modification of the Kjeldahl method (57).

Sulphate determinations were carried out by digesting a sample of the material (50 - 150 mg.) in boiling 8N hydrochloric acid (containing 1 - 2 drops of concentrated nitric acid) for several hours, diluting the filtered solution and estimating sulphate gravimetrically as its barium salt.

Acetyl determinations were carried out as described in Belcher and Godbert (57).

Methoxyl contents were determined by a volumetric Zeisel method.

Moisture/alcohol contents were determined by weighing samples before and after drying at 40°/0.3 mm. over phosphorus pentoxide for 24 hours.

Residual alcohol was estimated by the method of Newman (59). After drying for 24 hours at 40°/0.3 mm. a weighed sample was mixed with anhydrous sodium sulphate. Evacuation of the system for 15 minutes at 50° removed the alcohol which was absorbed into standard acid dichromate solution. The excess dichromate was then determined by the addition of excess potassium iodide and titration with 0.025N sodium thiosulphate solution in the

presence of sodium bicarbonate.

Ash. The quantity of ash was determined by ignition to constant weight of a weighed quantity of the material in a platinum or a silica crucible. Sulphated ash was determined by repeated dropwise treatment of the ash with concentrated sulphuric acid and ignition until constant weight was attained.

Small scale hydrolyses were carried out by treating samples of the material (ca. 40 - 100 mg.) in sealed tubes with N sulphuric acid for 4 hours at 100°. The hydrolysates were neutralised in the cold with barium carbonate and the filtrates evaporated to syrups which were deionised by repeated concentration and extraction with alcohol.

Paper chromatograms were run with control sugars on Whatman No.1 paper at a constant temperature of 20°. The principal solvents were:

(A) Butanol/Ethanol/Water (5:4:3) upper phase.

(B) Ethyl Acetate/Pyridine/Water (10:4:3).

(C) Benzene/Butanol/Pyridine/Water (1:5:3:3) upper phase ("Tate and Lyle").

Papers were sprayed with saturated aniline oxalate solution and the spots developed at 120°.

Rates of movement of sugars on the chromatograms are described in terms of the following ratios:

R_F = Distance travelled by sugar.
" " " solvent front.

R_G = Distance travelled by sugar.
" " " 2:3:4:6-tetra-O-methyl glucose.

R_{Gal} = Distance travelled by sugar.

" " " galactose.

The following abbreviations have been used:

G = galactose, A = arabinose, x = xylose, Gl = glucose and R = rhamnose.

Electroionophoresis experiments were carried out on a water-cooled apparatus constructed as described by Foster (62); Whatman No.1 paper was used in a borate buffer at pH 10, one hour being allowed for equilibration. After running for 4 hours (500 V, 12.5 mA) the paper was air dried and the sugars located by either aniline oxalate (+ 5% acetic acid) spray at 100° or, in the case of the polysaccharide, by treating with toluidine blue reagent (58).

Quantitative analysis of a Sugar Solution. (60). A definite quantity of the sugar solution was accurately delivered on to the chromatography paper (Whatman No.1) with an Agla semi-micro syringe and the paper was irrigated with solvent (C) for 2 or 3 days. It was then dried for 24 hours in vacuo and the sugars located by spraying side and central strips with ammoniacal silver nitrate (61). The sugars were then eluted with cold water from the appropriate strips of the chromatogram and the eluants (ca. 5 ml.) heated with 0.25 M sodium metaperiodate solution for 20 minutes at 100° in stoppered tubes (8" x 1"), the upper parts of which were cooled by condenser coils. Ethylene glycol (0.3 ml.) was added to the cooled solution and the formic acid produced was estimated with 0.01N carbonate-free sodium

hydroxide solution using methyl-red indicator. Water and paper blanks were treated simultaneously.

Demethylations were carried out on partly methylated derivatives by treatment with hydrobromic acid in a sealed tube at 100° for 5 - 10 minutes, followed by neutralisation with silver carbonate and deionisation of the filtrate by alcohol extraction. The free sugars produced were identified chromatographically.

Extraction of the Polysaccharide.

Two samples of the filamentous green seaweed *Cladophora rupestris* have been used in these investigations. Sample I was collected at Dunbar in July, 1953, from rockpools mid-way between high and low tide. This was dried at 60° - 80° for 16 hours and milled to a fine powder. Sample II was collected at Dunbar in November of the same year, dried at 60° - 80° for 16 hours and used as the whole weed.

I. Slow Extraction of Sample I.

(1) Slow Extraction.

A portion of the powdered weed (800 g.) was treated with 0.01N hydrochloric acid (4 l.) in a mechanical shaker for 48 hours. The solid material was removed on the centrifuge and washed twice with water. The centrifugate and washings were evaporated to a viscous solution (*ca.* 500 ml.) which was added dropwise with vigorous mechanical stirring to alcohol (4 l.). The precipitate of grey fibrous material was filtered, washed with alcohol and ether and dried. This was termed the cold extract (C.E.). Yield 115 g., i.e. 14.4% of dry weed. The residue, freed from all traces of acid, was then extracted four times with water (8 l.) for 24 hour periods with mechanical stirring on a boiling water bath. The extracts were filtered hot through muslin and the filtrates, after centrifugation and evaporation to small bulk, were precipitated into a large excess of alcohol. The four precipitates so obtained were termed the hot extracts (H.E.). Total yield 42 g., 5.3%.

(2) Sodium Carbonate Extraction.

The material was finally extracted for a 12-hour period with sodium carbonate solution (0.5%; 4 l.). The filtrate was

concentrated to about one fifth of the volume and hydrochloric acid was added during further concentration to maintain the pH between 7 and 8. The viscous solution was finally poured into alcohol and a grey fibrous material was precipitated. (Yield 5.0 g.).

II. Quick Extraction of Sample II.

The whole weed (500 g.) was first freed from extraneous matter by washing with cold running water for 12 hours and, after removal of some of the colouring matter by three treatments with cold alcohol (2.5 l.), was dried in vacuo (3 days). Recovery {after allowance for moisture/alcohol (14%) and residual alcohol contents (0.6%)} 272 g., amounting to a loss of weight of 45%. A sample of this dried material (43 g.) was extracted with boiling water (4 l.) for 3 hours, filtered through muslin and the residue reboiled with water (2.5 l.) for a further hour. After evaporation of the combined filtrates one portion of the viscous solution was freeze-dried and gave a white flaky material, while the remainder was precipitated into alcohol. This gave a cream coloured powder. (Total yield, 4.9 g., i.e. 11.4% calculated on the weight of cleaned, dried alcohol-extracted weed). The residual weed was washed with alcohol and ether and dried in vacuo (37.6 g.). The polysaccharide obtained by this method was termed the quick hot extract (Q.H.E.).

III. Extraction by Lead Complex Method.(51).

A portion of Sample II (100 g.) after washing with water and treatment with alcohol as described for Q.H.E. (2), was extracted for 3 hours with boiling water (5 l.) and re-extracted with water

for a further 2 hours. The combined filtrates were evaporated to small bulk and treated with neutral lead acetate solution. After removal of the precipitate by filtration through "filtercel", the solution was treated with barium hydroxide solution until alkaline to phenolphthalein. The precipitated lead hydroxide-carbohydrate complex was separated on the centrifuge and decomposed by shaking overnight, with 1.2N sulphuric acid; the mixture was dialysed for a week against running water, centrifuged free from extraneous solid, concentrated to small bulk and precipitated into excess alcohol. Yield, 1.43 g. Ash 12%; (as sulphate) 23.2%. This amounted to a recovery of only 1.43%.

Nitrogen Contents of Extracts.

Extract.		%N
1. Cold (C.E.)		2.1
Hot (H.E.)	1st Extract	2.9
	2nd Extract	3.1
	3rd and 4th Extract	1.8
Sodium Carbonate		4.6
2. Quick Hot (Q.H.E.) Freeze-dried product		3.4
Alcohol precipitated		3.0
3. Lead complex		1.0 (4)

By a direct Kjeldahl distillation, without prior digestion, the extract was found to be entirely free from ammonium salts.

Furthermore no amino-sugars, such as D-glucosamine, could be detected on chromatographic analysis of the syrup obtained from the hydrolysis of various extracts, but spraying with Ninhydrin reagent revealed the presence of alanine and other amino acids. The hydrolysates of all the fractions in all the extraction procedures described on the previous pages gave identical chromatograms, indicating the presence of galactose, glucose, arabinose, xylose and rhamnose. Purification methods were carried out on samples of the H.E. and C.H.E.

Investigation of Methods for Purification of Extracts.

1. Filtercel-Animal Charcoal. An aqueous solution of the H.E. (1.98 g.) was refluxed gently for one hour with a mixture of filtercel and animal charcoal (1:1). After centrifuging off the solid matter, the solution was evaporated to small bulk and precipitated into alcohol. Recovery: 1.21 g., loss 39%. The extract was unchanged in appearance and in nitrogen content.

2. Alcohol Extraction. No extraction of colouring matter from the H.E. took place either by standing over cold alcohol for 24 hours or by refluxing the mixture for one hour.

3. Freeze-drying. Freeze-drying an aqueous solution of the H.E. or C.H.E. produced an off-white, light, flaky material more readily soluble than the alcohol precipitated extract.

4. Cadmium Complex. (63). Attempts to substantially reduce the nitrogen content by precipitation of protein as the cadmium complex were largely unsuccessful. Variations in

experimental conditions e.g. concentrations of H.E., cadmium sulphate, sodium hydroxide, did not improve the results. Typical procedures were as follows:

(a) An aqueous solution of the H.E. (N, 3.0%; 403 mg., 100 ml.) was treated with cadmium sulphate (200 mg.) on a boiling water-bath and N sodium hydroxide solution (2 ml.) was added dropwise with mechanical stirring. The precipitate was centrifuged and the process repeated three times. The solution, after dialysis overnight, was freeze-dried. Recovery: 12 mg. i.e. 3%; N, 1.6%.

(b) A second sample (503.9 mg., 50 ml.) was subjected to a single precipitation with cadmium sulphate (1 g.) and N sodium hydroxide solution (10 ml.), centrifugation, dialysis and freeze-drying as before. Recovery: 44 mg., i.e. 8.7%; N, 1.3%.

5. Copper Complex.(64). An aqueous solution of copper chloride (50 g., 200 ml.) was added slowly, with vigorous mechanical stirring, to an aqueous solution of the H.E. (325 mg.). Concentrated ammonia solution was then added slowly until the light blue of the copper hydroxide precipitate changed to the deep blue, characteristic of the cuprammonium ion. The precipitate was filtered through a sintered glass filter and washed with ethanol (200 ml.), 5% ethanolic hydrogen chloride until free from copper, with ethanol until chloride-free, and finally with ether. Recovery: 100.6 mg. i.e. 31%; N, 2.9%.

6. Fehling's solution. A concentrated solution of the H.E. was treated with an equal volume of Fehling's solution. No precipitation occurred even on standing.

7. Zinc sulphate - sodium hydroxide. (65). N Sodium hydroxide solution was added dropwise to a solution (50 ml.) containing H.E. (400 mg.) and zinc sulphate (400 mg.). It was found that this process precipitated all the contents of the solution.

8. Ammonium Sulphate. Attempts were made to precipitate protein from solutions of the H.E. at full and half saturations with ammonium sulphate. Under these conditions carbohydrate and protein were both precipitated.

A further sample of H.E. (150 mg., 20 ml. water) was treated with ammonium sulphate (1 g.) and, after standing for 8 days, the brown fibrous material which separated was centrifuged off. The filtrate was passed down alternate columns of Amberlite resins IR. 4B - OH and IR 100-H until free from inorganic ions. The material was finally recovered by freeze-drying.
Recovery : 5.4 mg., i.e. 3.6%; N, 2.2%.

9. Basic Lead Acetate. Addition of a saturated solution of basic lead acetate to a solution of the H.E. at 60° gave only slight precipitation; no further separation was observed on standing.

10. Picric Acid. An aqueous solution of the H.E. (400 mg., 60 ml.) was saturated with picric acid and kept at 0° for 2 days. The solid material was then filtered off and the clear solution dialysed for 7 days, evaporated to small bulk and precipitated into alcohol. Recovery: 86.6 mg., i.e. 22%; N,1.97%.

11. Phosphotungstic Acid. An aqueous solution of H.E. (400 mg., 50 ml.) was mixed with the acid (2.5 g.). After standing, the mixture was treated as in 10.
Recovery: 253.5 mg., i.e. 63%; N, 1.18%. However, incomplete removal of the phosphotungstic acid was shown by the toluene-3:4-dithiol test (66). Furthermore, apart from the difficulty of removing the precipitant, the method offers no advantage over the trichloracetic acid method (see below).

12. Phosphomolybdic Acid. The acid (2.5 g.) was dissolved in an aqueous solution of the H.E. (400 mg., 50 ml.). Subsequent treatment as in 10 gave 127.8 mg., i.e. 32%; N, 1.54%.

13. Debasing Resin. An aqueous solution of the H.E. (N, 1.4%; 145 mg., 80 ml.) was passed slowly through a column of Zeocarb 215. The acid solution was dialysed first against concentrated solutions of calcium chloride until neutral (2 days) and then against running water for 1 day. After concentration, it was precipitated into alcohol.
Recovery: 70.3 mg., i.e. 49%; N, 1.23%.

14. An attempt was made to reduce the nitrogen content by treatment with proteolytic enzymes (papain, pepsin) but difficulty was encountered in freeing the polysaccharide material from the enzymes.

15. Trichloracetic Acid. (67). It was found that a 4% solution of this acid constituted the most economical and efficient protein precipitant. Its effectiveness has been examined at various scales of working, the general procedure always being as follows:

To an aqueous solution of the H.E. (1 - 1.5%) was added a concentrated solution of the acid containing the requisite weight to make a final 4% solution. On standing at room temperature for several days, the very fine precipitate was centrifuged off (Sharples Centrifuge 12,000 r.p.m.) and the clear solution dialysed to neutrality (3 weeks) and isolated by freeze-drying or alcohol precipitation. The polysaccharide was recovered in 60 - 65% yield with nitrogen values within the range 1.4 - 1.6%. The N content was not further reduced by a second treatment with the acid at 4% concentration and experiments at various other concentrations (4 - 10%) and also subsequent treatment with basic lead acetate failed to reduce this figure. Reduction, however, could be effected by fractional precipitation of the polysaccharide by addition of alcohol. After removal of the first fractions, the residual solution was evaporated to small bulk and precipitated into excess alcohol to give the most highly purified polysaccharide as an off-white powder. A typical fractionation is given below.

Alcohol Concentration.	Yield.	N Content.	$[\alpha]_D^{20°}$ (c., 0.1 in water).
50%	0.25 g.	2.06%	+ 91.5°
70%	1.53 g.	1.80%	+ 98.0°
Residual	4.25 g.	1.26%	+ 69.0°
	6.03 g.		

16. Sevag Treatment. (68). A solution of the H.E. (180 mg.; N, 1.3% after trichloracetic acid treatment) was shaken with a mixture of chloroform (0.25 vol.) and n-butanol (0.10 vol.) for 30 minutes. After centrifuging, the upper aqueous layer was decanted off and subjected to three further similar treatments. The aqueous solution was finally freeze-dried.
Recovery: 41 mg., i.e. 23%; N, 1.05%.

17. A sample of acetylated polysaccharide (acetyl content 20.6%; N, 1.4%, see page 62) was saponified by warming in dilute alkali. The alkaline solution was dialysed free from extraneous inorganic material, evaporated to small bulk and freeze-dried. N, 1.6%.

Final Extraction Procedures Adopted.

Large Scale Extraction. A portion of the whole weed, (Sample II, 1200 g.), after washing with water and treatment with alcohol as for the H.E. (2) (page 22), was extracted for 3 hours in boiling water (50 gal.) and the residue re-extracted for $1\frac{1}{2}$ hours in water (30 gal.). The clarified extracts were combined and evaporated to a neutral green gel (ca. 2 gal.) which was broken by the addition of trichloracetic acid to 4% concentration. Subsequent treatment as before (page 28) gave the polysaccharide in two fractions, the first by precipitation with 40% alcohol (31 g., N, 1.51%) and the remainder by precipitation, after concentration in excess alcohol (10 g.; N, 1.19%).

Dilute Acid Extraction. The weed (450 g.) which had been pre-treated by washing with a cold 0.25% aqueous solution of sodium carbonate (3 l.), acetone (2 x 2 l.) and finally

alcohol (3 l.), was extracted for 4 hours at 70° at a pH 3 - 4 (hydrochloric acid). Treatment with trichloracetic acid (page 28) gave a light-coloured powder (29 g., N, 1.39%).

Hydrolysis and Chromatography of the Fractions.

Each of the extracts and fractions of the extracts and each of the products from the various purification procedures gave identical chromatograms after hydrolyses with acid (N sulphuric acid at 100° for 4 hours, and in certain cases, for 36 hours, and 4 N sulphuric acid for 5 hours at 100°). Spots corresponding to galactose, glucose, arabinose, xylose and rhamnose were obtained in every sample examined, in, as far as could be judged visually, the same relative proportions. No trace of any spot corresponding to glucosamine or a uronic acid could be detected on running in basic and in acid solvents, nor could any indication of ketose material be obtained. Ninhydrin spray reagent, however, indicated the presence of several amino-acids including alanine.

Examination of the Cold Extract (C.E.) and of the Sodium Carbonate Extract.

Hydrolyses and chromatography of the C.E. and sodium carbonate extracts both indicated the presence of galactose, glucose, arabinose, xylose and rhamnose in, as far as could be judged visually, the same relative proportions as were found for the hot water extracts. Chromatograms of the hydrolysates run in butanol-acetic acid-water (4:1:5) solvent indicated the complete absence of uronic acid. In view of these facts, and

of the more efficient quick hot (Q.H.E.) and acid extraction procedures developed, these extracts were not further investigated.

General Properties of the Polysaccharide.

All further work was carried out on samples of the polysaccharide of the following constitution; N, 1.26% $[\alpha]_D^{20°}$ +.69° (c., 1.0 in water); CH_3CO, nil; OMe, nil (see page 17); ash (direct) 13.7%; (as sulphate) 16.1%; SO_4, 19.6% (see page 17). This value was not reduced by prolonged dialysis of an aqueous solution of the polysaccharide against running water. While cations such as calcium could be precipitated, no sulphate could be detected in solutions of the polysaccharide until after hydrolysis. An aqueous solution of the polysaccharide was non-reducing to Fehling's solution and gave a faintly reddish colour on treatment with iodine solution.

Qualitative analysis of the ash revealed the presence of SO_4", Ca^{++}, Fe^{+++}, Al^{+++}, Na^{+}, K^{+} and SiO_2, and the metals were estimated quantitatively by dissolving a sample of the sulphated ash (112.2 mg.) in concentrated hydrochloric acid containing two drops of concentrated nitric acid. The solution was filtered free from a small amount of insoluble material and the iron present was determined by addition of ammonia solution, and ignition of the precipitated ferric hydroxide to constant weight in a porcelain crucible (69). The filtrate from this operation was evaporated to small bulk and made up to a 100 ml. The calcium was determined on 30 ml. of this solution by precipitation as the oxalate. This was redissolved

in dilute sulphuric acid and titrated at 60° with 0.026N potassium permanganate solution (70). The sulphate present in the remaining 70 ml. of solution was determined as its barium salt in the usual way. Calcium and excess barium were removed from the solution by precipitation as their carbonates and the filtrate made up to 250 ml. Sodium was determined on a 50 ml. portion as sodium zinc uranyl acetate (71). Potassium was estimated on a second 50 ml. portion, after volatilisation of ammonium salts, as perchlorate (72). The results are tabulated below.

Ion.	Percentage of Ash.	Percentage of Polysaccharide.
$SO_4^{"}$	62.0	10.0
Ca^{++}	22.1	3.5
Fe^{+++}	4.4	0.7
Na^{+}	3.7	0.6
K^{+}	0.4	
Insol. ($Al_2O_3 + SiO_2$)	6.1	
	98.7	

Hydrolysis and Chromatographic Analysis.

(a) Qualitative: Chromatographic analysis of the hydrolysate (N sulphuric acid, 4 hours) showed galactose, glucose, arabinose, xylose and rhamnose.

(b) Quantitative: The polysaccharide (95.7 mg.) after hydrolysis (N sulphuric acid, 4 hours at 100°), addition of ribose (12.56 mg.), neutralisation and deionisation, gave a syrup (62.56 mg.) which was made up to 10 ml. with water (Solution A). Application of an accurately measured volume of this solution (0.4720 ml.) to a chromatogram and estimation as described on page 19 gave:

Sugar.	Vol. of 0.01N Sodium Hydroxide Solution (ml.) (blank 0.070).	Estimated Weight of Sugar in 10 ml. of Solution (mg.).	Proportional %age of Sugars in syrup.	Molecular Proportions.
galactose	1.829	13.9	38.0	2.8
glucose	0.139	1.1	3.0	0.2
arabinose	1.940	15.4	42.0	3.7
xylose	0.527	4.2	11.5	1.0
rhamnose	0.137 - 0.321	1.2 - 2.8	3.3 - 7.7	0.4 (Av. value).
ribose	1.372	(10.9)		
Total (less ribose):		36.6		

A second determination gave concordant results but the rhamnose gave variable results within the range shown above.

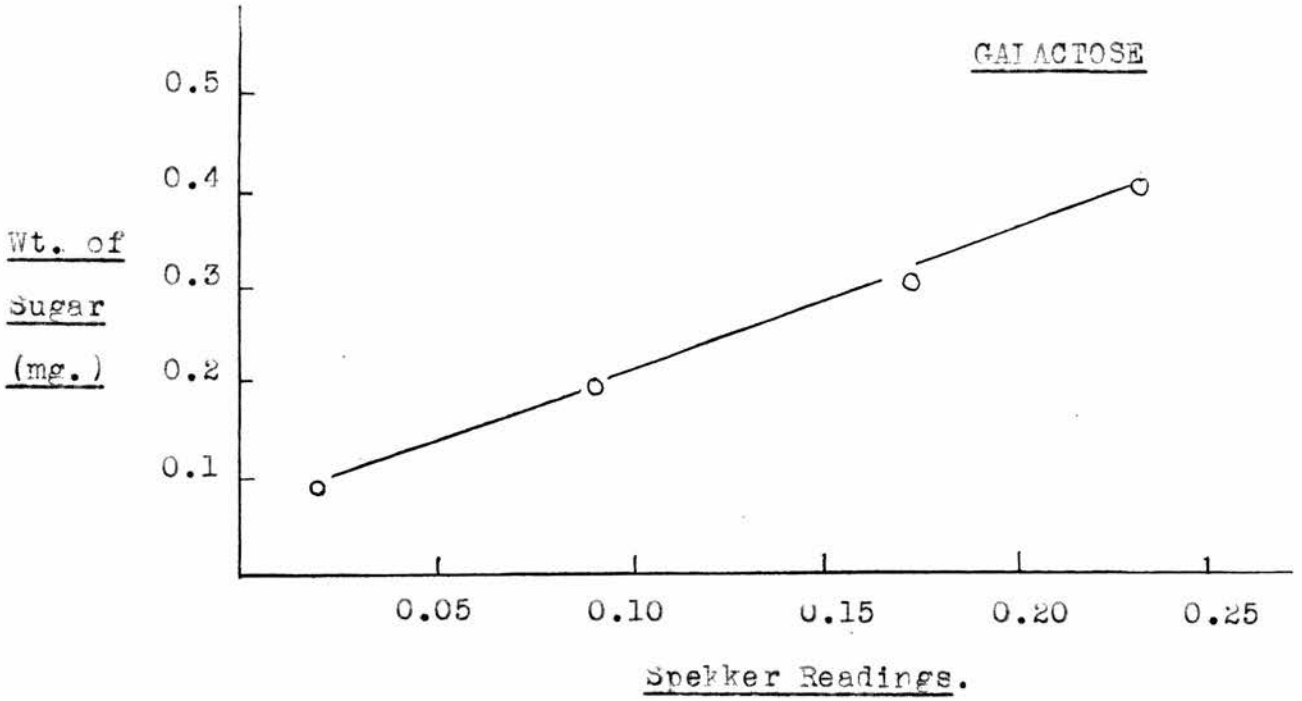
Percentage absorption of ribose = 8.8%

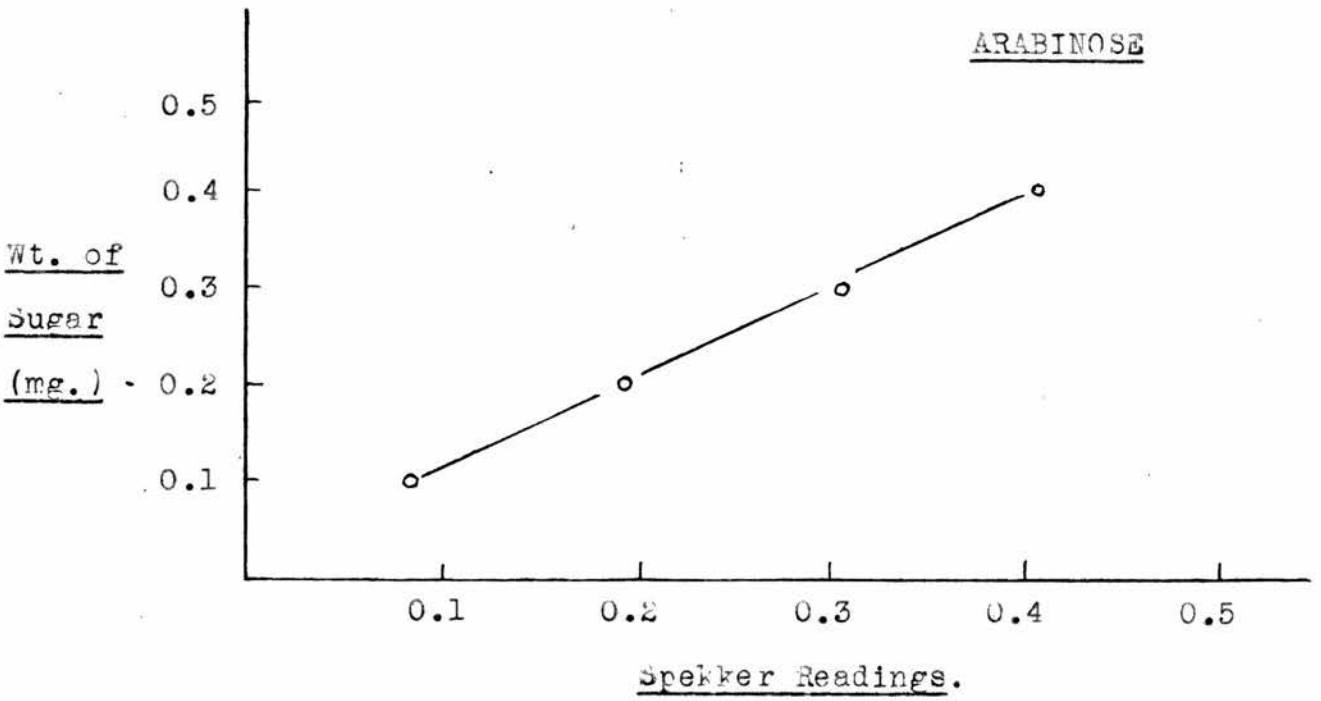
Assuming same degree of absorption for each sugar:

Total weight of 5 sugars in 95.7 mg. polysaccharide	=	39.6 mg.
" " " protein " " "	=	7.5 mg.
" " " sulphate " " "	=	15.7 mg.
" " " metals " " "	=	5.8 mg.
		68.6 mg.

Weight of polysaccharide accounted for = $\frac{68.6}{95.7} \times 100$ = 72%

The galactose and arabinose present in the solution A of the syrup were also estimated by the Nelson-Somogyi method (73). The sugars were located on the chromatograms and eluted as before, and, in order to equalise the amounts of dissolved oxygen, each of the solutions (ca. 5 ml.) were heated in a boiling water bath for 15 minutes with a series of standard solutions containing 0.1 mg., 0.2 mg., 0.3 mg., and 0.4 mg. of the appropriate sugar; water and paper blanks were treated simultaneously. After cooling and addition of the copper reagent (1 ml.) the solutions were heated at 100° for 20 minutes. Arsenomolybdic acid solution (2 ml.) was added and each sample was made up to 25 ml. in a standard flask. The intensity of the blue-green colour which developed was measured in a Spekker electrophotometer with "green 604" filters. The readings were plotted against sugar concentrations and the linear relationships so obtained enabled the weights of sugar in the unknown solutions to be determined, after comparison with the reading of the paper blank in the instrument.


GALACTOSE
Wt. of Sugar (mg.)
0.5
0.4
0.3
0.2
0.1
0.05
0.10
0.15
0.20
0.25
Spekker Readings.


ARABINOSE
Wt. of Sugar (mg.)
0.5
0.4
0.3
0.2
0.1
0.1
0.2
0.3
0.4
0.5
Spekker Readings.

Readings of Standard Solutions of Galactose and Arabinose.

Amount of Sugar (mg.).	Galactose.	Arabinose.
0.100	0.018	0.082
0.200	0.091	0.198
0.300	0.172	0.306
0.400	0.232	0.404

Readings of Galactose and Arabinose in Solution A.

Volume of Solution A spotted on each chromatogram = 0.1200 ml.

Galactose gave the following readings:

(1) 0.059 corresponding to 0.155 mg. of sugar.

(2) 0.062 " " 0.160 mg. " "

Arabinose gave the following readings:

(1) 0.148 corresponding to 0.160 mg. of sugar.

(2) 0.154 " " 0.170 mg. " "

Weight of galactose in Syrup A = 13.3 mg. (cf. 13.9 mg. by periodate method).

" " arabinose " " " = 14.2 mg. (cf. 15.4 mg. by periodate method).

(c) Uronic Acid and Amino-Sugars. No trace of either of these entities could be detected in the syrups produced from the polysaccharide by mild hydrolysis or by hydrolysis under much more severe conditions, i.e. 36 hours N sulphuric acid at 100° and 5 hours 4N sulphuric acid at 100°. {Solvents (B), (C) and butanol/acetic acid/water (4:1:5), aniline oxalate and napthoresorcinol spray reagents}.

(d) Ketonic and Anhydro Sugars.

(i) Oxalic acid hydrolysis. A portion of the polysaccharide was hydrolysed with 0.5N oxalic acid solution for 6 hours at 100° under carbon dioxide. The mixture was neutralised with calcium carbonate, and the filtrate evaporated in a stream of carbon dioxide. Chromatograms of the deionised syrup so produced were sprayed with aqueous and alcoholic solutions of urea oxalate and also with anthrone reagent (74) but no indication of either breakdown or ketonic products was obtained. Spraying with aniline oxalate solution showed the usual five sugars together with slower moving oligosaccharides.

(ii) Anthrone reagent (74). An aqueous solution of the polysaccharide along with solutions of fructose and various other polysaccharides were spotted on a paper. This was sprayed with anthrone reagent and heated at 110°. The results are tabulated below:

Carbohydrate Source.	Colour of Spot.
Triticin	Intense yellow brown.
Fructose	" " "
Chondrus Crispus	Faint yellow brown.
Polysiphonia fastigiata	" " "
Gelidium lactifolium	" " "
Agar	" " "
Cladophora rupestris	No visible spot.

(iii) Seliwanoff Test. (75). Addition of the oxalic acid hydrolysate to the reagent (resorcinol in concentrated hydrochloric acid) produced a reddish colour on standing

overnight, whereas a blank run concurrently remained colourless. On prolonged standing (2 days) the reagent itself developed a red colour.

Hydrolysis of Polysaccharide followed Polarimetrically.

The polysaccharide (76.1 mg.) was hydrolysed at 100° with N sulphuric acid (60 ml.). 10ml. samples were withdrawn at intervals and their rotations determined. Specific rotations were calculated on the basis of the original concentration of the solution.

Time (hrs.):	0	1	2	3	4	5
$[\alpha]_D^{20°}$	+66°	69°	75°	79°	79°	79°

Removal of Sulphate Residues by Alkaline Hydrolysis.

1. The polysaccharide (520.6 mg.) was treated at 100° with N sodium hydroxide solution (100 ml.). Samples were withdrawn at intervals and the sulphate liberated was estimated as the barium salt. The percentage hydrolysis was calculated on an initial sulphate content of 19.6%.

Time of Hydrolysis (hrs.).	Percentage Hyd. of Sulphate.
$\frac{1}{2}$	12.0
4	58.5
6	65.0
8	68.0

2. The polysaccharide (1.123 g.) was treated with N potassium hydroxide solution (100 ml.) at 95° in an atmosphere of nitrogen. A portion of the solution (30 ml.) was removed after 4 hours' treatment and the remainder after 8 hours. Both samples were dialysed free from extraneous ions against running water, evaporated to small bulk and freeze-dried. A previous attempt to isolate the degraded polysaccharide by alcohol precipitation instead of freeze-drying was unsuccessful owing to incomplete precipitation.

Fraction.	Recovery.	Percentage Recovery.	SO_4" content.	N content.
4 hour	182 mg.	54%	15.1%	0.69%
8 hour	400 mg.	51%	13.2%	0.60%

Both fractions gave reddish-brown colours on standing

for some time in Seliwanoff reagent, a blank remaining colourless. The original polysaccharide under similar conditions gave no colour. The chromatogram of the hydrolysates of the two alkali degraded polysaccharides gave spots corresponding to galactose, glucose, arabinose, xylose and rhamnose in their usual intensities.

Periodate Oxidation of the Alkali-Degraded Polysaccharide.

It was found that the periodate treatment (0.025 M; 48 hours) of a sample of alkali-degraded polysaccharide (8 hours, N alkali at 100°) resulted in a product which on hydrolysis and chromatographic analysis showed the almost entire elimination of xylose and a reduction in the amount of galactose. The chromatogram was in fact identical to that produced by the oxypolysaccharide (see page 49).

Large Scale Hydrolysis of the Polysaccharides and Separation of the Sugars on a Cellulose Column.

The polysaccharide (9 g.) was hydrolysed by 0.5 N oxalic acid (400 ml.) for 6 hours at 100° in an atmosphere of carbon dioxide. The cooled solution was neutralised with calcium carbonate and the filtrate and washings evaporated to dryness in a current of carbon dioxide. After further drying (phosphorus pentoxide overnight) the residue was extracted with absolute ethanol and the extracts evaporated to a syrup which was re-extracted. This was repeated until the syrup was free from inorganic ions. A final cold water extraction and evaporation gave a clear, light yellow syrup 2.94 g., $[\alpha]_D^{20°}$ + 59.3° (c., 1.7 in H_2O). Kjeldahl estimations on this material and the use of Ninhydrin reagent showed the absence of nitrogen containing products. Chromatographic examination of the syrup indicated galactose, glucose, arabinose, xylose and rhamnose together with small amounts of oligosaccharides.

A cellulose column 85 x 2.7 cm., prepared by the method of Hough, Jones and Wadman (76), was washed with water, n-butanol and finally with n-butanol two-thirds saturated with water, with which solvent the column was subsequently irrigated. The syrup (2.01 g.) was dissolved in the minimum quantity of aqueous butanol and applied dropwise by pipette to the top of the column. The eluant was allowed to percolate down from a constant head reservoir and the liquid issuing at the lower end was collected in 5 ml. fractions on an automatic fraction

cutter adjusted to change at 32 minute intervals.

Every tenth tube was evaporated and analysed chromatographically; those tubes containing the same sugar were combined along with their water washings and evaporated to dryness. Initial purification of the fractions was effected by water extraction and filtration which removed a considerable amount of wax-like contaminant.

After 1900 tubes had been collected the solvent was changed from butanol two-thirds saturated with water to butanol saturated with water + 5% ethanol. After 2,670 tubes had been collected the column was washed with water (6 l.). The following fractions were obtained:

Tubes.	Fraction.	Colour of Spot.	R_F	Weight in mg. after purification.
1 - 100	-			
101 - 325	I	pink	0.78	58.2
326 - 344	-			
345 - 396	II	yellow	varies but identical with rhamnose	81.2
397 - 449	II→III			7.0
450 - 530	III	pink head/ yellow tail	0.39	40.7
531 - 544	III→IV			7.4
545 - 670	IV	pink	0.39	232.2
671 - 689	IV→V			3.3
690 - 880	V	pink	0.34	585.5
881 - 1000	V→VI			5.3
1001 - 1170	VI	brown	0.29	75.7
1171 - 1215	VI→VII			8.6
1216 - 1555	VII	brown	0.24	513.6
1556 - 2410	VII→VIII			10.0
2411 - 2670	VIII	pink	0.13	143.0
			Total =	1771.7 mg.

Recovery: 93.5%. Chromatograms were developed in solvents (B) and (C) (page 18) and R_F values are related to the latter.

Examination of the Fractions.

Fraction I. Syrup, 58.2 mg., $[\alpha]_D^{20°}$ + 20.8° (c., 0.9 in H_2O)
n_D 1.4875.

This gave a strongly positive Seliwanoff test, a bright red colour developing about one minute after addition of the reagent.

Fraction II. 81.2 mg. This was purified by extraction with water (2 x 4 ml.). Filtration and evaporation gave a colourless syrup which crystallised on standing: $[\alpha]_D^{19.5°}$ + 8.8° (c., 1.2 in water) conversion to the crystalline benzoyl hydrazone by treatment with a saturated alcoholic solution of benzoyl hydrazine (77) characterised the sugar as L-Rhamnose. M.p. of benzoyl hydrazone 179 - 181° (decomp.) undepressed by admixture with an authentic specimen.

Fraction III. Syrup, 40.7 mg. This syrup gave a positive Seliwanoff test.

Fraction IV. 232.2 mg. After purification and treatment with activated charcoal, this fraction gave colourless crystals from methanol. The physical constants corresponded to α-D-Xylose; m.p. 144 - 144.5° (undepressed by admixture with an authentic specimen). $[\alpha]_D^{19°}$ + 41.0° → +16.0°, (c., 0.9 in water).

The identity of the sugar was further established through the preparation of its dibenzylidene dimethyl acetal derivative (78) by treatment with a solution of benzaldehyde in methanolic hydrogen chloride. This derivative after recrystallisation from methanol had m.p. and mixed m.p. with an authentic specimen 210 - 211°.

Fraction V. 585.5 mg. After purification as described for xylose, the syrup crystallised as colourless needles from methanol m.p. 154 - 154.5°, $[\alpha]_D^{20°}$ + 156° → + 107°, (c., 1.3 in water).

Found; C, 39.9; H, 6.5

Calc. for $C_5H_{10}O_5$; C, 40.0; H, 6.7%

The sugar was finally characterised as β-L-Arabinose by conversion to the benzoyl hydrazone (79). After recrystallisation from water, this had m.p. and mixed m.p. 199 - 203° (decomp.).

Fraction VI. 75.7 mg. After several treatments with activated charcoal the sugar crystallised from methanol, m.p. 148 - 150°, $[\alpha]_D^{18°}$ + 55.5°, (c., 0.8 in water). Its identity as D-Glucose was established by aerobic oxidation to gluconic acid by the specific enzyme, glucose oxidase (notatin) (80). The rate of oxygen uptake was measured in a Warburg apparatus.

Fraction VII. 513.6 mg. After purification as for the other fractions, the syrup crystallised from methanol, m.p. 165 - 166°, $[\alpha]_D^{19°}$ + 138° → + 83.5°, (c., 1.2 in water). Its identity as α-D-Galactose was further established by the preparation of the diethylmercaptal derivative (81). Recrystallisation twice from absolute alcohol gave feathery white crystals, m.p. and mixed m.p. with an authentic specimen 140 - 141°.

Found; C, 41.8; H, 7.6; S, 21.9

Calc. for $C_{10}H_{22}O_5S_2$: C, 41.8; H, 7.7, S, 22.4

Fraction VIII. Syrup, 143.0 mg. This fraction consisted of a mixture of oligosaccharides; the predominant one gave a pink spot on the chromatogram and had an R_F value of 0.13.

As the separation and purification of the oligosaccharides produced by mild acid hydrolysis of the polysaccharide is dealt with later in the work, this fraction was not further investigated.

Attempted Fractionation of the Polysaccharide.

1. Alcohol Fractionation. The various fractions of the polysaccharide precipitated from aqueous solution at different concentrations of alcohol (50%, 70%, 85% and 95%) gave identical chromatograms on hydrolyses and each fraction had the same sulphate content (19.1%, 19.8%, 19.2%, 19.9% respectively).

2. Electrophoresis Experiments. A 1% solution of the polysaccharide in borate buffer pH 10 gave a Schlieren diagram with a symmetrical peak. Electroionophoresis experiments on the polysaccharide and oxypolysaccharide were also carried out with Whatman No.1 paper in the same borate buffer for 4 - 5 hours at 500 V. Spraying with an alcoholic solution of toluidine blue, (58) a reagent specific for sulphate groupings, gave a single blue spot on the chromatogram. A number of other spray reagents were investigated {aniline oxalate - phosphoric acid, periodate permanganate (82), ammoniacal silver nitrate (61), periodate and Schiff's Reagent (83) and lead tetracetate in benzene (83)}, but only toluidine blue gave positive results.

3. Potassium Chloride Fractionation. (27, 28). A portion of the polysaccharide was converted to its sodium salt by dialysis against concentrated sodium chloride solution, then against running

water till free from extraneous ions, followed by precipitation into alcohol. An aqueous solution of this material (200 ml., 0.24%) was treated with stirring at room temperature with sufficient solid potassium chloride to give a 0.25 molar solution. No precipitation occurred immediately or after standing for 4 days nor was any precipitate obtained on repeating the experiment at 0.15, 0.50 and 0.66 molar concentrations of potassium chloride.

4. Enzymatic Hydrolysis of the Glucan. A dilute aqueous solution of the polysaccharide in a sodium acetate - acetic acid buffer at pH 5.0 was incubated at 35° with laminarinase, an enzyme specific for β - 1:3 - and β - 1:4 - linked glucans. Samples of the solution were spotted on a chromatogram initially and after 0.5 hour, 1 hour, 1.5 hours and then at 2 hourly intervals, an enzyme blank being run concurrently. The papers were irrigated with solvent (B) and sprayed with ammoniacal silver nitrate reagent (61). A single spot corresponding to glucose began to appear after 2 hours. The enzyme blank gave no spots on the chromatogram.

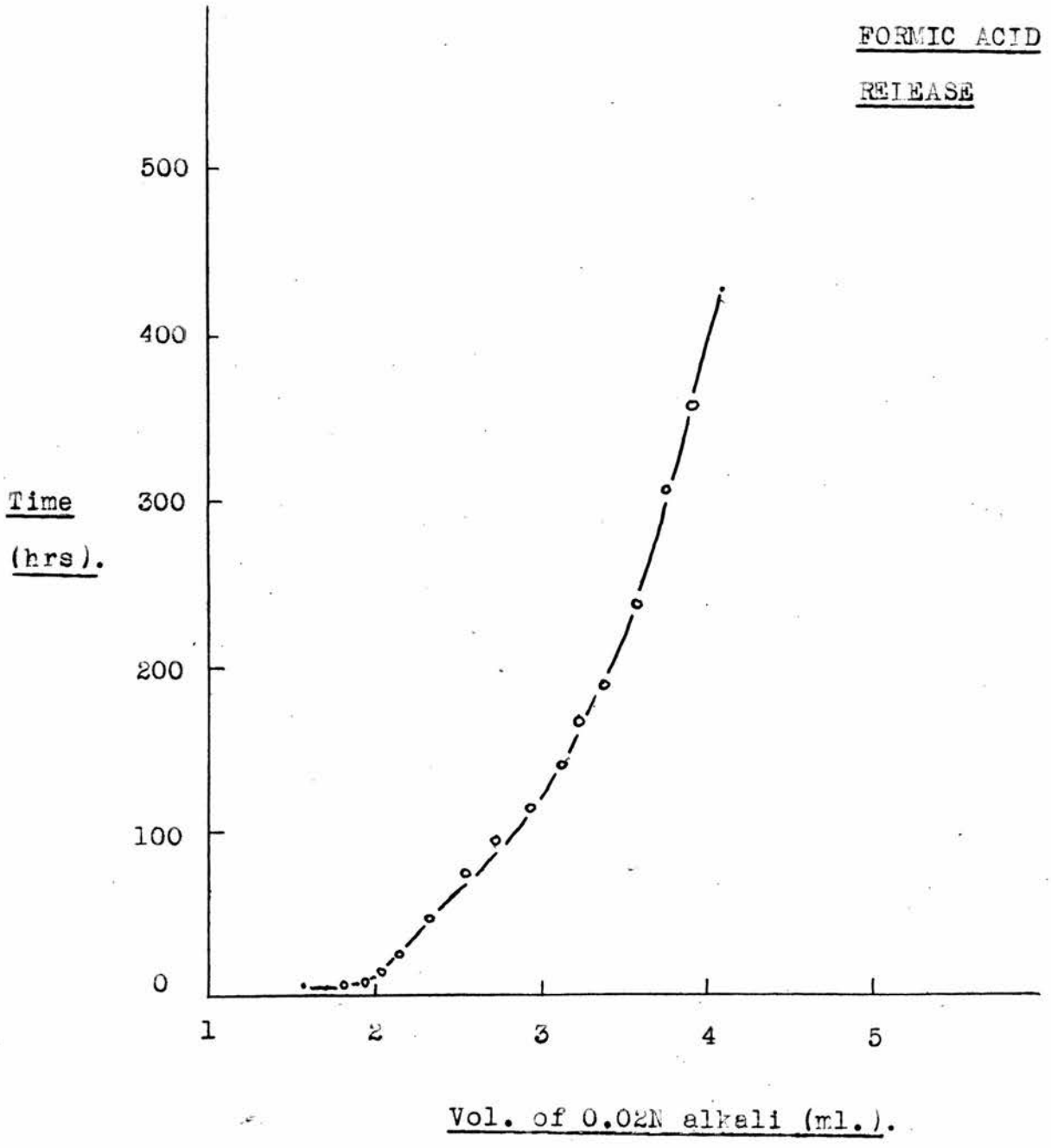
5. The separation of a glucan by chloroform extraction of the acetylated polysaccharide is described on page 66.

Periodate Oxidation of the Polysaccharide.

1. Periodate Uptake. An aqueous solution of the polysaccharide sodium salt prepared as described on page 45, (467.6 mg., 60 ml.), was treated with 0.1 M sodium metaperiodate solution (50 ml.) a blank being run simultaneously. After 36 hours the periodate uptake was determined by the method of Fleury and Lange (84) by treating 20 ml. portions of the solution with 40 ml. 0.1 N sodium arsenite solution together with sodium bicarbonate and potassium iodide. After standing for 15 minutes in the dark, the excess arsenite was determined by titration with 0.1 N iodine solution, using starch indicator. (It was found later that a more satisfactory end-point could be obtained without recourse to this indicator). Concordant results were obtained.

1 mole periodate ≡ 347 g. polysaccharide.

2. Formic Acid Release. An aqueous solution of the polysaccharide (3.9 g., 160 ml.) was treated with an equal volume of 0.26 M sodium metaperiodate solution. Samples (2 ml.) were removed at intervals, excess periodate destroyed by addition of ethylene glycol and the formic acid released measured by titration with 0.02 N (carbonate-free) sodium hydroxide solution.


FORMIC ACID
RELEASE
Time
(hrs).
500
400
300
200
100
0
1
2
3
4
5
Vol. of 0.02N alkali (ml.).

Time (hrs.).	Vol. of 0.02N alkali (ml.).	Time (hrs.).	Vol. of 0.02N alkali (ml.).
2	1.59	114	2.94
5	1.83	138	3.12
8	1.96	162	3.20
14	2.02	186	3.36
22	2.16	234	3.60
48	2.32	306	3.71
72	2.54	354	3.90
92	2.74	426	4.10

Acid was released continuously and no definite break in the curve was obtained.

Hypoiodite Oxidation of the Polysaccharide.

An aqueous solution of the polysaccharide (159 mg., 10 ml.) in a sodium hydroxide - borate buffer at pH 11.40 (85) was treated with 20 ml. 0.05 N iodine solution. After standing for 6 hours, the solution was acidified with 10 ml. 4 N sulphuric acid and the residual iodine titrated with 0.02 N sodium thiosulphate solution. A blank was run simultaneously.

100 mg. polysaccharide ≡ 0.064 ml. 0.1 N iodine solution

i.e. 2 litres N iodine solution ≡ 31,200 g. polysaccharide

A second determination gave concordant results.

Periodate Oxidation and Isolation of the Oxypolysaccharide.

The oxypolysaccharide was first isolated from its aqueous sodium metaperiodate solution (48 hours' treatment) by dialysis of the mixture against running water till free from extraneous ions (3 - 4 weeks), evaporation of the solution and freeze-drying. However, in order to avoid the prolonged periods necessary for dialysis, it was found more convenient to destroy periodate and iodate by passing sulphur dioxide gas into the solution and precipitating the oxypolysaccharide by addition of alcohol. The precipitate, after solution in water and reprecipitation in alcohol till free from inorganic contaminants, was finally washed with alcohol and ether and dried in vacuo. Recoveries from both methods were of the order: 80 - 87%; SO_4", 20.2%; N, 1.3%.

Investigation of the Oxypolysaccharide.
Hydrolysis and Estimation of the Sugars.

Hydrolysis of the oxypolysaccharide and chromatographic analysis of the resultant syrup showed that only a trace of xylose remained and that the proportion of galactose present was also reduced. Quantitative estimation of the sugars present in the hydrolysate, according to the method described on page 19 gave:

Sugar.	Proportional %age in Oxypolysaccharide.	Proportional %age in Polysaccharide.
galactose	16.6	38.0
glucose	6.6	3.0
arabinose	61.4	42.0
xylose	Too small to estimate.	11.5
rhamnose	10.2 - 15.4	3.3 - 7.7

Alkali Degradation.

The oxypolysaccharide (1.8205 g.) was treated with N sodium hydroxide solution (100 ml.) at 90° in an atmosphere of nitrogen. Part of the solution (50 ml.) was removed after 1 hour and the remainder after 3 hours. Both samples were dialysed to neutrality, evaporated and freeze-dried.

Sample.	Recovery.	%age Recovery.	SO_4"	N
1 hour	387.0 mg.	43%	17.6%	0.9%
3 hours	334.3 mg.	37%	14.1%	0.5%

As far as could be judged visually, chromatograms of the hydrolysates of these materials were identical with that of the oxypolysaccharide.

Partial Hydrolysis of the Polysaccharide and of the Oxypolysaccharide.

1. Mild Hydrolysis with Sulphuric Acid.

(a) Small Scale. Samples of the polysaccharide and oxypolysaccharide were hydrolysed in sealed tubes with 0.01 N sulphuric acid at 90° for 3 hours. Neutralisation, deionisation and chromatographic analysis of the resulting syrup gave the following spots:

Sugar.	Polysaccharide.	Oxypolysaccharide.
galactose	strong	very faint
glucose	nil	nil
arabinose	faint	strong
xylose	strong	very faint
rhamnose	nil	nil

The chromatograms also showed a number of slower moving spots corresponding to oligosaccharides.

(b) Large Scale. Samples of the polysaccharide and oxypolysaccharide (ca. 350 mg.) were hydrolysed with 0.04 N sulphuric acid for 3 hours at 90°. The hydrolysates were dialysed against distilled water and the dialysates neutralised with barium carbonate and deionised by alcohol extraction in the usual way. Chromatography of the resulting syrups showed, in the case of the polysaccharide, galactose and xylose together with a trace of arabinose. The syrup from the oxypolysaccharide,

however, showed only a general streaking down the paper which, after further treatment with N sulphuric acid for 3 hours at 100°, gave clean chromatograms showing rhamnose and galactose at about the same intensity, together with a smaller amount of arabinose. Chromatograms were also treated with toluidine blue reagent but no trace of sulphated sugar was found.

The solutions remaining in the dialysis bag was further dialysed against running water till free from impurities and then evaporated and freeze-dried. The residual polysaccharide (X) (79 mg. i.e. 24% of starting material) was hydrolysed and found to contain all five sugars, whereas the residue from the oxypolysaccharide (Y) (43 mg. i.e. 13% of starting material), SO_4 19.95%, contained principally arabinose together with much smaller amounts of galactose and glucose. An electroionophoretogram (4 hours 500 V.; borate buffer pH 10) of the oxypolysaccharide residue (Y) gave a single spot on treatment with toluidine blue reagent. Further treatment of this material with sodium metaperiodate solution and hydrolysis of the product gave a chromatogram which, as far as could be judged visually, was identical to that from the residue (Y) before further periodate treatment. It was found however that more vigorous acid treatment of the oxypolysaccharide (0.07 N and 0.1 N sulphuric acid for 3 hours) gave residual materials (Z) which appeared to have an increased galactose content relative to the arabinose.

(c) Increase in Acidity of Solution during Mild Hydrolysis.

The polysaccharide (200 mg.) was treated at 100° with 0.014 N sulphuric acid (20 ml.). Portions (5 ml.) were removed

after 3 hours and 7 hours and the increase in acidity determined by titration with 0.02 N alkali solution:

Time.	Normality.
0	0.014 N
3	0.018 N
7	0.021 N

2. Hydrolysis with Oxalic Acid.

(a) With 0.1 N Oxalic Acid Solution at Room Temperature.

The polysaccharide (400 mg.) was treated with 0.1 N oxalic acid solution (100 ml.) at room temperature for 1 month. After neutralisation, chromatography showed that only a trace of galactose had been liberated.

(b) With 0.1 N Oxalic Acid Solution at 50°.

The polysaccharide (2% solution) was treated with 0.1 N oxalic acid solution at 50° in an atmosphere of carbon dioxide. Samples were removed at intervals, cooled and neutralised with calcium carbonate and analysed chromatographically:

Sample.	Sugars liberated.	Seliwanoff Test.
½ hour 2 1 hour	Trace of galactose.	Negative.
2 hours 3 hours	Galactose, xylose arabinose with traces of oligo-saccharides.	Slight. Very strong.
4 hours 5 hours	Galactose, xylose with increased relative quantities of arabinose and oligosaccharides.	Strong. Moderate.
9 hours	The above sugars together with glucose and rhamnose.	Moderate.

(d) With 0.5 N Oxalic Acid Solution at 95° and Isolation of the Residual Polysaccharide.

(i) Small Scale. The polysaccharide (772.8 mg.) was hydrolysed with 0.5 N oxalic acid solution at 95° for 3 hours. The solution was dialysed to neutrality, evaporated and freeze-dried. (Recovery: 87.4 mg., i.e. 11.2%; N, 3.0%). Hydrolysis and chromatographic analysis of this residue showed galactose, glucose and arabinose in about the same relative intensities, together with some rhamnose. Attempts to repeat this experiment gave anomalous results.

(ii) Large Scale. The polysaccharide was hydrolysed with 0.5 N oxalic acid solution (12 g., 400 ml.) and dialysed as described above. The dialysate was neutralised and deionised to give a clear yellow syrup (B) (10 g.) which contained all the usual sugars together with a series of oligosaccharides. This syrup (B) was first subjected to a preliminary rough separation on a charcoal-celite (50:50) column.

Fraction I. This syrup (4 g.) consisted of the monosaccharides together with a fast moving oligosaccharide (pink spot to aniline oxalate) and only traces of other oligosaccharides.

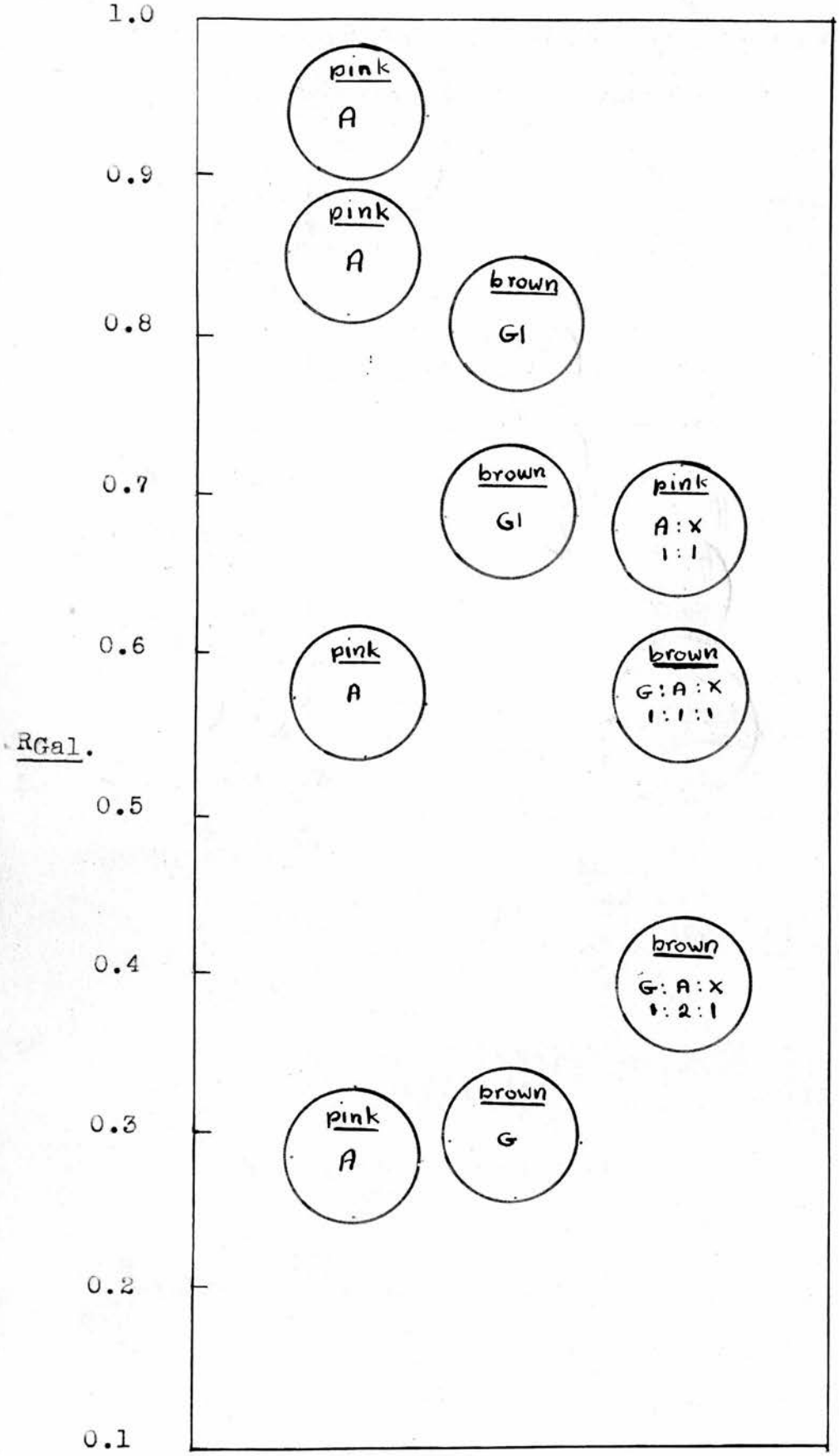
Fraction II. This syrup (521.3 mg.) consisted of a series of oligosaccharides contaminated with small quantities of the monosaccharides.

Separation of Fraction II on a Carbon-Celite Column.

The column was packed according to the method of Bacon and Bell (86). B.D.H. acid washed charcoal (40 g.) was mechanically

mixed with "Celite No.535" (40 g.; Johns Mansville Co., Ltd., London, S.W.1). The mixture was made into a slurry with water (250 ml.) and poured onto the column under gentle suction. The base was supported by a plug of glass wool surmounted by a layer (2 cm.) of celite. This gave a carbon-celite column of the dimension 3 x 40 cm.

After washing the column to neutrality with water (2 l.), Fraction II, dissolved in water (10 ml.), was applied to the top of the column and allowed to soak in over a period of 1 hour. The initial eluant was water containing 1% ethanol to give an initial column speed of 30 ml./hour and the ethanol concentration was increased by 1% at 24 hour intervals. The eluants were collected in 7 ml. portions on an automatic fraction cutter set initially at a 16-minute interval change, and latterly at a 32-minute change as the speed of elution decreased. Every tenth tube was evaporated and analysed chromatographically {Solvent (B) overnight}, like fractions being combined. Each of these combined fractions was purified by alcohol extraction and since considerable overlapping and streaking had occurred each was further separated from contaminants by paper chromatography; the weights given in the table represent those of the purified syrups. The column was finally washed with 50% aqueous ethanol (4 l.). The whole operation was carried out at a constant temperature of 20° and care was taken to prevent undue vibration of the column.



A = arabinose

X = xylose

G = galactose

Gl = glucose

CHROMATOGRAPHIC DIAGRAM OF OLIGOSACCHARIDES.

The rates of travel of the spots are related to galactose as a standard ($R_{Gal.}$) and the proportions of monosaccharides produced on hydrolyses were estimated visually.

Fraction.	Weight (mg.) after separation on paper.	Colour of Spot (Aniline Oxalate).	$R_{Gal.}$	Sugars on Hydrolysis and Proportions.
1 - 220	174.6	Monosaccharides and trace of slower moving brown spot.		
221 - 280	4.0	Pink.	0.58	Arabinose.
281 - 340	4.7	Pink.	0.68	Arabinose:Xylose. (1:1)
341 - 420	8.9	Brown.	0.38	Arabinose: Galactose: Xylose. (2:1:1)
421 - 435	5.7	(a) Brown.	0.29	Galactose.
		(b) Brown.	0.69	Glucose.
436 - 510	6.5	Brown.	0.58	Galactose: Arabinose:Xylose. (1:1:1)
511 - 609	Complex mixture, not worked up.			
610 - 750	6.0	Pink.	0.28	Arabinose.
751 - 810	Nil.			
811 - 920	3.4	Brown.	0.81	Glucose.
921 - 1275	Nil.			
50% aqueous ethanol (2 l.)	Nil.			

Fraction 811 - 920 was found to be chromatographically and electroionophoretically identical with laminaribiose. It also moved at an identical rate to an authentic specimen of this disaccharide in the form of their benzylamine complexes (87).

Charcoal Column Treatment of Synthetic Mixture of Sugars.

In order to ensure that the oligosaccharides were not artefacts, a synthetic mixture of sugars (galactose 3.8 g.; arabinose 4.2 g.; xylose 1.2 g.; rhamnose 0.5 g.; total 9.9 g.) was treated with 0.5 N oxalic acid solution (400 ml. + 1 ml. N sulphuric acid) in precisely the same way as described for the hydrolysis of the polysaccharide and isolation of Syrup (B). Yield 8.7 g. This syrup (C) was dissolved in water and allowed to percolate down the charcoal column used in the oligosaccharide separation. After standing in the column overnight at 20°, graded elution was commenced, the initial rate of elution being 30 ml./hour.

Fraction.	Eluant.	Weight.	Sugars on Chromatogram.
C_1	Water (500 ml.)	8.3 g.	All monosaccharide. No trace of oligosaccharides.
C_2	10% aqueous ethanol (500 ml.)		
	20% aqueous ethanol (500 ml.)	0.2 g.	"
	50% aqueous ethanol (2 l.)		

Separation of Fraction I (page 54) on a Cellulose Column.

The syrup (2.7 g.) was separated on a cellulose column 85 x 2.7 cm., and was eluted with butanol saturated with water. After removal of the monosaccharides, the oligosaccharides were

eluted with ethanol. Two oligosaccharides were obtained. The main component gave a descrete pink spot with aniline oxalate and had an $R_{Gal.}$ value of 0.87 while the faster moving minor constituent gave a diffuse pink spot $R_{Gal.}$ 0.95. These were separated by paper chromatography {Solvent (B)}. Both gave only arabinose on hydrolysis and methyl arabopyranoside on methanolysis and neither moved at the same rate on the chromatogram as 3-O-β-L-arabopyranosyl-L-arabinose $R_{Gal.}$ 0.78, the only available specimen of an authentic arabinose disaccharide.

Barry Degradation Experiments.

The formation of complex compounds between the oxypolysaccharide and phenylhydrazine and isoniazid (isonicotinoylhydrazine) according to the method of Barry (88) has been investigated.

1. Phenylhydrazine. An aqueous solution of the oxypolysaccharide (598 mg., 10 ml.) on treatment with excess phenylhydrazine acetate in phenylhydrazine solution gave an immediate yellow-brown precipitate. After heating the mixture on a boiling water bath for 1 hour, this solid material (A) was removed, washed with alcohol and ether, and dried. Yield: 47.5 mg; N, 6.2%. On standing at room temperature for 3 days, the mother liquors deposited a pale yellow precipitate (11.2 mg.) and further precipitation occurred on addition of alcohol.
Yield: 97.9 mg.; N, 5.8%. Total yield: 156.6 mg. i.e. 26%.

A quantity of this material (620 mg.) was treated with excess of phenylhydrazine acetate reagent on a boiling water bath for 15 minutes. After cooling, the solution was diluted with 3 times its own volume of ethanol and allowed to stand for 2 days at room temperature. The resulting precipitate was then centrifuged off, washed free of colouring matter and dried. Recovery: 36.5 mg; N, 6.9%. The hydrolysate of this material gave a chromatogram identical with that from the oxypolysaccharide (page 49).

The neutral alcoholic solution which remained was evaporated to dryness and extracted with ether. The solid residues gave, on recrystallisation from water, colourless

crystals of β-acetyl-phenylhydrazine m.p. 127°, undepressed by admixture with an authentic specimen. The ether extracts were washed with dilute acetic acid, water, sodium bicarbonate solution and water and evaporated to a red oil, which was dissolved in benzene and separated on an alumina column. Two red bands were isolated which, on evaporation, gave red oils which failed to crystallise.

2. Phenylhydrazine (Cold). An aqueous solution of the oxypolysaccharide (2.8063 g., 100 ml.) was treated as before with excess of the phenylhydrazine reagent. A yellow scum immediately formed which became granular on addition of alcohol (90 ml.). This was filtered off, washed with alcohol and ether and dried to a yellowish powder (259.9 mg.; N, 3.7%). Addition of more alcohol to the solution produced a further pale-yellow precipitate which was isolated as before (1.0857 g.; N, 3.6%). The hydrolysates of these two fractions were found to be chromatographically identical with that of the oxypolysaccharide.

The alcoholic filtrates from these reactions were dialysed against alcohol/acetone (50:50) to give a dialysate (a) and a clear red solution (b) remaining in the dialysis bag.

(a) Treatment of the Dialysate. The combined solutions were evaporated to dryness, the residue dissolved in ether and the ethereal solution washed with dilute acetic acid, water, sodium carbonate solution and water. Removal of the ether gave a red oil which was separated on alumina. One red band was isolated which, on evaporation, gave a red oil which failed to crystallise.

(b) Treatment of the Residue. The solution remaining in the dialysis bag was dialysed for a week against running water. This led to the precipitation of a finely divided brownish material (149.6 mg.; N, 5.9%). This substance gave a red solution in alcohol. The remaining clear aqueous solution was evaporated to small bulk and freeze-dried to a brown-coloured solid (7.6 mg.). The hydrolysates of both these fractions were chromatographically identical with that of the oxypolysaccharide.

3. Condensation with Isoniazid. An aqueous solution of isoniazid (800 mg., 8 ml.) was added to an aqueous solution of the oxypolysaccharide (387.1 mg., 40 ml.) and after standing for 1 hour, the clear solution was acidified with acetic acid (0.5 ml.). A canary-yellow precipitate was at once produced (96 mg.; N, 6.6%) and a second precipitate (13.6 mg.) deposited after standing for 3 days. Hydrolysis and chromatography of these precipitates showed the usual sugars in the same relative proportions as in the oxypolysaccharide.

Treatment of the Polysaccharide with Phenylhydrazine.

The polysaccharide (330 mg.) was treated with phenylhydrazine acetate in phenylhydrazine at 100° for 20 minutes. On cooling, excess alcohol was added and the resulting precipitate washed with alcohol and ether and dried. Recovery: 301.2 mg. The chromatogram of the hydrolysate of this material was identical to that of the original polysaccharide.

Acetylation of the Polysaccharide.

A number of acetylation procedures were investigated including the dispersion of the polysaccharide in pyridine both directly and by means of a pyridine-water azeotrope, followed by treatment with acetic anhydride, and the method finally adopted was to disperse the polysaccharide (20 g.) in formamide (150 ml.) in an "Ato-Mix" at room temperature and then treat with pyridine (1,000 ml.) and acetic anhydride (500 ml.). After allowing the mixture to stand for 2 weeks at room temperature, the clear, brown solution was diluted with ice-water and dialysed for 2 - 3 weeks. The acetylated polysaccharide was then isolated by concentration of the aqueous solution and freeze-drying. (Yield: 17.6 g.; CH_3CO, 17.2%). This material could now be dispersed to a clear, brown jelly directly in pyridine (70 ml.) at room temperature in the "Ato-Mix". Acetic anhydride (60 ml.) was added and, after standing at room temperature for a week, the product was isolated by dialysis and freeze-drying. (Yield: 14.5 g.; CH_3CO, 19.4%). A third acetylation in pyridine (50 ml.) and acetic anhydride (40 ml.) as before, gave a recovery of 11.0 g. (CH_3CO, 20.6%; N, 1.4%). Neither a fourth acetylation on a portion of the product nor acetylation carried out at higher temperatures increased the acetyl content. Hydrolysis, followed by chromatographic analysis of the material showed the presence of galactose, glucose, arabinose, xylose and rhamnose in their usual proportions. A gelatinous precipitate was obtained by pouring the reaction mixture into water and the acetylated polysaccharide could also be

precipitated directly from the acetylation mixture in a large excess of acetone - ether (50:50) but difficulty was encountered in removing the last traces of impurity from the product.

Methylation of the Polysaccharide.

1. Simultaneous De-acetylation and cold Haworth Methylation.

The acetylated polysaccharide (10 g.; CH_3CO, 20.6%) was dispersed in water (400 ml.). Dimethyl sulphate (110 ml.) and 40% sodium hydroxide solution (120 ml.) were then added simultaneously and dropwise with mechanical stirring over a period of 6 hours. The temperature throughout was maintained below 10° and the reactions were carried out in an atmosphere of nitrogen. Further quantities of dimethyl sulphate (110 ml.) and 40% alkali (120 ml.) were added dropwise daily for five days and the reaction mixture, after dialysis for 10 days against running water, was evaporated to small bulk. The methylation was repeated twice at room temperature. After a final dialysis for 3 weeks the solution was evaporated to small bulk and freeze-dried to an off-white product.
Yield: 6 g., OMe 19.6%.

2. Attempted Methylation by Dimethyl Formamide. (89).

Freeze-dried polysaccharide (1.6 g.) was dispersed in dimethyl formamide and shaken for 60 hours with methyl iodide (10 ml.) and silver oxide (10 g.). The silver residues were filtered off and the filtrate and washings treated with further quantities of methyl iodide (10 ml.) and silver oxide (10 g.) during 24 hours. After filtration of silver salts, colloidal

silver was brought into the solution by addition of potassium cyanide. The clear solution was dialysed till free from impurity, evaporated and freeze-dried.
Recovery: 0.5 g. (31%); OMe 4.1%.

3. Haworth Methylation (45°). Dimethyl sulphate (20 ml.) and 30% sodium hydroxide solution (50 ml.) were added dropwise at 45° over 4 hours to an aqueous solution of the partially methylated polysaccharide (OMe 19.6%; 2 g., 30 ml.). The temperature was maintained at 45° for a further 2 hours and then raised to 70° for 2 hours, the whole operation being carried out with mechanical stirring and in an atmosphere of nitrogen. After neutralisation with glacial acetic acid, the solution was dialysed for 10 days and concentrated. The methylation was repeated twice. Final dialysis and freeze-drying gave a white, hygroscopic solid.
Yield: 634.2 mg., i.e. 32%; OMe 25.2%.

4. Purdie Methylation. Partly methylated polysaccharide (OMe 19.6%; 3 g.) was gently refluxed with methyl iodide (120 ml.) containing 10% anhydrous methanol. Silver oxide (15 g.) was added in small portions over 6 hours and, after refluxing for a further 24 hours, the methyl iodide was removed and the solid residues extracted with aqueous acetone. Colloidal silver in the evaporated extract was dissolved by addition of potassium cyanide and removed by dialysis. Freeze-drying gave a pure white product, 900 mg. i.e. 30%; OMe 24.0%. Two further Purdie methylations did not increase the OMe content beyond 25.4%.

5. Thallium Methylation. (90). A solution of N thallous hydroxide (5 ml.) was added to an aqueous solution of the partly

methylated polysaccharide (OMe 19.6%; 1 g., 10 ml.). The mixture was freeze-dried to a yellow solid which, after being crushed to a fine powder, was suspended in methyl iodide (150 ml.) and the mixture refluxed till the solution no longer gave an alkaline reaction (4 days). These processes were carried out either in the dark or in subdued light. The methyl iodide was removed by distillation and the solid residues extracted with cold water. The extracts were evaporated and re-extracted with water, this process being repeated till the product was free from inorganic impurity. Freeze drying gave a pure white hygroscopic solid. Yield; 494.6 mg., i.e. 49%; OMe 25.2%. A second thallium methylation did not increase the methoxyl content.

6. Dimethyl Formamide Methylation. The procedure described under (2.) was repeated on a sample of the partly methylated polysaccharide (OMe 19.6%; 154.0 mg.). The product (46.1 mg.) had OMe 24.6%.

Large Scale Acetylation and Methylation of Polysaccharide and Removal of the Glucan.

The polysaccharide (19 g.) was acetylated 3 times as described on page 62 and the acetylated derivative (17.8 g.; CH_3CO, 20.3%) was extracted with chloroform in a Soxhlet apparatus. The chloroform extracts were evaporated to dryness and the solid residue washed with hot water and purified by cold chloroform solution (3 x 10 ml.), filtration and evaporation to dryness. The chloroform soluble acetylated material was then saponified by warming in dilute alkali and the solution was dialysed to neutrality, concentrated and freeze-dried. The product (51.2 mg.) on hydrolysis and chromatographic analysis showed glucose together with traces of galactose, arabinose and a very faint indication of xylose. Rhamnose was not visible on the chromatogram. The chloroform insoluble acetate (16.5 g.) contained galactose, arabinose, xylose and rhamnose in their usual proportions, but was entirely devoid of glucose.

Periodate Oxidation of the Glucan.

The glucan (SO_4"< 3%; N, nil.) gave no colour with iodine. Periodate uptake was measured by the method of Fleury and Lange (84).

1 mole periodate ≡ 420 g. glucan.

Methylation of the Chloroform-Insoluble Acetate.

The glucose-free acetylated polysaccharide (16.5 g.) was methylated four times with sodium hydroxide and methyl sulphate, twice in the cold, and twice at 45° as described in the previous sections. Yield: 6.9 g.; OMe 25.1%; N, 0.60%; SO_4", 16.2%; $[\alpha]_D^{21°}$ + 92° (c., 4.0 in water); ash (as sulphate) 8.3%. This material was water-soluble but also, to a slight extent, chloroform-soluble. The hydrolysate of the chloroform extract (OMe 26.1%) was chromatographically indistinguishable from that of the chloroform insoluble residue (OMe 24.8%).

Methanolysis and Hydrolysis of the Methylated Polysaccharide.

The methylated polysaccharide (6 g.) was treated with 5% methanolic hydrogen chloride (200 ml.). After refluxing for 6 hours, the solution, which was too dark in colour for polarimeter readings, was neutralised with silver carbonate and the filtrate and washings were evaporated to a syrup. This was further treated with 0.5 N hydrochloric acid (200 ml.) for 3 hours at 95° and again neutralised with silver carbonate. Deionisation by treatment of the filtrate with hydrogen sulphide and Amberlite resins (IR. 100 - H and IR. 4B - OH) gave a syrup which was further purified by 3x extraction with alcohol. Yield: 4.18 g.

Separation of the Hydrolysate.

The syrup (3.8 g.) was applied to the top of a cellulose column, 85 x 2.7 cm., and eluted with light petroleum (b.p. 100 - 120°) - butanol mixture (7:3) saturated with water. Fractions were collected in 7 ml. portions by means of the automatic fraction cutter and every tenth tube was evaporated and analysed chromatographically, like fractions being combined. After 1,500 tubes had been collected, the solvent was gradually altered to a mixture of butanol - light petroleum (b.p. 100 - 120°) (1:1) saturated with water and after 2,300 tubes, gradually to butanol saturated with water.

The weights of the fractions recorded in the table are those obtained after a preliminary purification by hot water extraction. Further purification was carried out by treatment with activated charcoal in either boiling alcohol or hot water, followed by alternate extraction and evaporation of the syrup with alcohol and water. Fractions varied in the amount of waxy contamination and in some cases considerable losses were involved in obtaining a sample pure enough for specific rotation measurements. The following fractions were obtained.

Tubes.	Fraction.	Colour of Spot.	R_G.	Weight (mg.).
1 - 80				-
81 - 120	I	grey	0.94	74.5
121 - 140				-
141 - 170	II	yellow-brown	0.88	30.9
171 - 195	II → III			12.2
196 - 210	III	yellow-green	0.88	23.7
211 - 230	III → IV			9.7
231 - 310	IV	pink	0.86	104.0
311 - 390	V	pink	0.74	58.2
391 - 570	VI	red	0.63	234.8
571 - 670	VII	yellow-green	0.57	78.4
671 - 690	VII → VIII			24.4
691 - 770	VIII	pink	0.54	49.3
771 - 1150	VII → VIII			115.7
1151 - 1500	IX	pink	0.38	539.3
1501 - 1570	IX → X			34.8
1571 - 1710	X	brown	0.38	32.2
1711 - 1835	X → XI			70.7
1836 - 2300	XI	pink	0.28	174.9
2301 - 2360	XI → XII			112.0
2361 - 2670	XII	brown	0.21	113.1
2671 - 2740	XII → XIII			201.7
2741 - 3050	XIII	pink	0.14	303.0
3051 - 3080	XIII → XIV			21.2
3081 - 3300	XIV	brown	0.08	498.2
Column washed.				Nil.

Total Recovery = 2.9 g.

%age Recovery = $\frac{2.9}{3.8} \times 100 = 76\%$

Investigation of the Fractions.

Fraction I. (74.5 mg.) $[\alpha]_D^{18°}$ + 4° (c., 1.4 in water).

This fraction was found to be a mixture of two components which gave spots on chromatographic analysis identical with authentic specimens of 2:3:5-tri-O-methyl arabinose (trace) and 2:3:4-tri-O-methyl xylose {Solvent (A) and benzene/ethanol/water (169:47:15)}. Separation on paper (benzene/ethanol/water) and further purification gave the xylose derivative (14 mg.) with $[\alpha]_D^{18.5°}$ + 13° (c., 0.7 in water) which value is in agreement with that for 2:3:4-tri-O-methyl-D-xylose, $[\alpha]_D$ + 18° (91).

Fraction II. (30.9 mg.). This fraction was chromatographically identical to an authentic specimen of 2:3:4:6-tetra-O-methyl galactose. Demethylation gave galactose as the only free sugar and, after further purification, the syrup (5.7 mg.) had OMe 49.8% (calc. for tetramethyl hexose OMe 52.5%), $[\alpha]_D^{19°}$ + 106° (c., 0.3 in water). Specific rotation values recorded for 2:3:4:6-tetra-O-methyl-D-galactose vary, but mostly fall within the range $[\alpha]_D$ + 114 → + 118° (92, 93).

Fraction III. (23.7 mg.). This fraction, which gave rhamnose on demethylation, corresponded on the chromatogram to a dimethyl rhamnose, the dimethyl derivatives of this sugar being chromatographically indistinguishable. Electroionophoresis experiments showed it to be a mixture, one component moving at an identical rate to that of an authentic specimen of 3:4-di-O-methyl rhamnose,

the second part remaining on the starting line. The rotation of the purified syrup (12.1 mg.) $[\alpha]_D^{18°}$ + 8°. (c., 0.6 in water) indicates that it is probably a mixture of 2:4 - di-O-methyl-L-rhamnose $[\alpha]_D$ - 19° (94) and 3:4 - di-O-methyl-L-rhamnose $[\alpha]_D$ + 18.6° (95) (equilibrium values). The 2:3 derivative has $[\alpha]_D$ + 42.5° (96).

Fraction IV. (104.0 mg.). Demethylation of this fraction gave galactose as the only free sugar. After further purification the syrup (60.2 mg.) had $[\alpha]_D^{18°}$ - 3.8° (c., 0.9 in water); OMe 40.1% (calc. for a trimethyl hexose OMe 41.9%). These results indicate that this fraction is a trimethyl galactose, which from rotation and chromatography data could be either 2:3:5 - tri-O-methyl-D-galactose $[\alpha]_D$ - 5° (97) or 3:4:6 - tri-O-methyl-D-galactose $[\alpha]_D$ - 4.3° (98). Treatment with bromine water, followed by neutralisation with silver carbonate, then by deionisation with hydrogen sulphide and distillation (0.01 mm.; 180° bath temp.), gave the lactone (12.7 mg.) $[\alpha]_D^{18°}$ -.29° (c., 0.6 in water). This corresponds to the lactone of 2:3:5 - tri-O-methyl-D-galactose $[\alpha]_D$ - 37° → - 32° (97); the lactone of the 3:4:6 derivative has $[\alpha]_D$ + 46.8° (98).

Fraction V. (58.2 mg.). This fraction, which gave xylose as the only free sugar on demethylation, was chromatographically and electroionophoretically identical with an authentic specimen of 2:3 - di-O-methyl xylose. After further purification, the

syrup (41.3 mg.) had $[\alpha]_D^{18°}$ + 20° (c., 1.1 in water) which corresponds to the value for 2:3-di-O-methyl-D-xylose $[\alpha]_D$ + 23° (equilibrium) (99). The material began to crystallise slowly on standing.

Fraction VI. (234.8 mg.). This fraction gave a single spot on the chromatogram, corresponding to 2:3:4-tri-O-methyl galactose but demethylation liberated a mixture of galactose and arabinose. No separation of these components could be achieved either by electroionophoresis experiments or by chromatography in solvents (A), (B) and (C). After further purification, the syrup had $[\alpha]_D^{18°}$ + 100° (c., 1.3 in water), OMe 38.6% (calc. for trimethyl hexose OMe 41.9% and for a dimethyl pentose 34.8%). From this evidence it seems possible that this fraction is a mixture of a tri-O-methyl-D-galactose, possibly the 2:3:4 - derivative $[\alpha]_D$ + 121° (equilibrium) (100) and a di-O-methyl-L-arabinose, possibly the 2:3 - derivative $[\alpha]_D$ + 107° (101).

Fraction VII. (78.4 mg.). This fraction, which was contaminated with the previous fraction, corresponded chromatographically and electroionophoretically to an authentic specimen of 4-O-methyl-L-rhamnose. Traces of contamination by the previous fraction were removed by separation on the paper chromatogram and purification of the syrup (27.2 mg.) gave $[\alpha]_D^{18°}$ + 15° (c., 1.4 in water) corresponding to the value recorded $[\alpha]_D$ + 13° (102). On standing, the syrup began to crystallise slowly.

Fraction VIII. (49.3 mg.). Arabinose was the only free sugar liberated on demethylation of this fraction. After further purification, the syrup (32.9 mg.) had $[\alpha]_D^{18°}$ + 101° (c., 1.6 in water). Conversion to the lactone as described for Fraction IV gave a syrup (4.9 mg.) $[\alpha]_D$ + 88° → + 40° (c., 0.4 in water) in 18 hours. These constants are in reasonable agreement with those of 2:4 - di-O-methyl-L-arabinose $[\alpha]_D$ + 118° (103), $[\alpha]_D$ of lactone + 99° → +39° (17 hours) (104).

Fraction IX. (539.3 mg.). This was indistinguishable chromatographically and electroionophoretically from an authentic specimen of 2-O-methyl-L-arabinose. Demethylation gave arabinose as the only free sugar and, after further purification the syrup had $[\alpha]_D^{18°}$ + 101.1° (c., 2.0 in water). Conversion of a portion (100 mg.) to the lactone as described for Fraction IV gave a syrup (22 mg) $[\alpha]_D$ - 38° (c., 1.1 in water). These constants are in reasonable agreement with those for 2-O-methyl-L-arabinose $[\alpha]_D$ + 100° (105); the lactone has $[\alpha]_D$ - 44° → - 40° (106).

Fraction X. (32.2 mg.). This fraction, on chromatography and electroionophoresis, corresponded to an authentic specimen of 2:4 - di-O-methyl galactose. Although contaminated with the previous fraction (both arabinose and galactose were liberated on demethylation), the purified syrup partially crystallised on standing. Recrystallisation from ethyl acetate gave a small crop of crystals m.p. 77 - 80°. Although the m.p. of 2:4- di-O-methyl-D-galactose is recorded in the literature as 98 - 99° (107),

the values obtained for the two available samples were 74 - 76° and 70 - 74°. Admixture with these samples caused no depression of m.p.

Fraction XI. (174.9 mg.). Arabinose was the only free sugar liberated on demethylation and it was found to be chromatographically and electroionophoretically identical with 3-O-methyl-L-arabinose. Further purification gave a syrup (51.5 mg.) with $[\alpha]_D^{18.5°}$ + 112° (c., 1.5 in water). Attempts to prepare the crystalline anilide derivative were unsuccessful but conversion to the lactone as described for Fraction IV gave a syrup (12.4 mg.) with $[\alpha]_D^{18°}$ - 65° (c., 0.6 in water). These constants are in reasonable agreement with 3-O-methyl-L-arabinose $[\alpha]_D$ + 110°, lactone $[\alpha]_D$ - 74° (108).

Fraction XII. (113.1 mg.). This fraction gave galactose as the only free sugar on demethylation and was chromatographically and electroionophoretically identical with 2-O-methyl galactose. After further purification, the syrup (30.3 mg.) had $[\alpha]_D^{17°}$ + 78° (c., 1.5 in water). Bromine oxidation and distillation as previously described gave the lactone (10.2 mg.) $[\alpha]_D^{18°}$ - 18° (c., 0.5 in water), which constants are in reasonable agreement with those of 2-O-methyl-D-galactose $[\alpha]_D$ + 80°, lactone $[\alpha]_D$ - 27° - - 24° (109).

Fraction XIII. (303.0 mg.). This corresponded to arabinose on the chromatogram. After further purification, it crystallised from methanol in colourless needles, m.p. 152 - 153.5°

(no depression on admixture with authentic specimen of L-arabinose) $[\alpha]_D^{18°}$ + 157° → +106° (c., 1.2 in water). The crystalline material was thus characterised as L-arabinose.

Fraction XIV. (498.2 mg.). This was chromatographically identical with galactose and, after further purification, crystallised from methanol m.p. 164 - 166° (no depression on admixture with authentic specimen of D-galactose) $[\alpha]_D^{19°}$ + 130° → + 81.2 (c., 1.4 in water) showed this fraction to be D-galactose.

DISCUSSION.

DISCUSSION.

Extraction of the Polysaccharide.

Extraction of both the whole and powdered weed with cold water, hot water and hot dilute sodium carbonate solution was investigated. The various extracts were hydrolysed and the hydrolysates all gave identical chromatograms. The extracts appeared to differ only in the nature and proportion of inorganic material present, in the amount of colouring matter and in their nitrogen contents (2.1 - 4.6%). The method of extraction finally adopted was to free the whole weed from extraneous matter by spraying with cold water, to remove some of the colouring matter by several treatments with cold alcohol and finally to extract the polysaccharide material with boiling water or dilute hydrochloric acid (pH 3 - 4) at 70°. The extracts were concentrated to a viscous solution from which the contents were isolated by alcohol precipitation or freeze-drying in an overall yield of 11% from the dried weed.

Neither ammonium salts nor amino-sugars could be detected in these extracts, but amino acids were shown by ninhydrin to be present in their hydrolysates. The amino acids were considered to originate from protein material which, in view of the nitrogen contents of the extracts (3 - 4%), would be present according to the factor 6.25 to the extent of 20 - 25%. The usual methods for protein removal were investigated. Attempts to precipitate protein as the cadmium complex, with basic lead

acetate, with picric, phosphotungstic and phosphomolybdic acids were all abortive. In some instances, protein precipitation was negligible while in others the polysaccharide and protein were both precipitated. The yields were poor and in no experiment was the nitrogen content reduced below 1.3%. Precipitation of the polysaccharide as the copper complex with copper chloride or with Fehling's solution failed to give any purification while treatment with zinc sulphate - sodium hydroxide led to complete precipitation of the contents of the solution. Extraction of the polysaccharide from the whole weed as the lead complex and subsequent decomposition of the complex gave a product N, 1.04%, but in very poor yield.

The most satisfactory and economical method was partial precipitation of the protein with 4% trichloracetic acid and alcohol fractionation of the clarified, dialysed and concentrated filtrate. After removal of impure fractions with 50% and 70% alcohol, a final fraction (N, 1.26%) was obtained and all further investigations were carried out on this material. It should be emphasised that the hydrolysates of all the fractions and products of purification were found to give identical sugar patterns on chromatographic analysis.

Treatment of the purified material by the Sevag method did effect a further slight reduction in nitrogen content (1.26 → 1.05%) but gave a very poor recovery, while treatment with hot alkali reduced the figure from 1.26% to 0.60% but caused considerable degradation of the carbohydrate material.

In view of these facts, it seems possible that this residual nitrogenous material may form an integral part of the polysaccharide molecule similar, for instance, to that of alginic acid (110) where the molecule appears to incorporate several amino acids or short peptide chains. It is also interesting to note in this connection that similar results were obtained in the case of Ulva lactuca (56), the only other sulphated polysaccharide from a green seaweed so far investigated. Here, the original extracts containing ca. 25% protein could be purified, albeit in poor yield, by the Sevag method to a minimum nitrogen content of 1.01%.

Properties of the Polysaccharide Extract.

This was obtained as an off-white non-reducing (to Fehling's solution) powder $[\alpha]_D^{20°}$ + 69° (c., 1.0 in water) which did not appear to contain ketose, amino-sugars, anhydro-bodies or uronic acid residues. It had OMe, nil; CH_3CO, nil; ash (as sulphate) 16.7% and gave a faintly reddish colour with iodine. A reddish colour also developed on standing overnight in Seliwanoff reagent but little significance can be attached to this reaction.

Hydrolysis of the extract was accompanied by only a small change in specific rotation from $[\alpha]_D$ + 66° → + 79°. The hydrolysate contained five sugars: L-arabinose, D-galactose, D-xylose, L-rhamnose and D-glucose in the molar proportions 3.7: 2.8: 1.0: 0.4: 0.2. Considerable difficulty was encountered, however, in obtaining concordant results for the estimation of rhamnose on the paper chromatogram and the figure quoted above must be regarded as an average figure from a number of estimations. These sugars occurred in all the extracts and in the purified materials in, as far as could be judged visually, the same relative proportions.

D-Glucose and D-xylose are common constituents of algal polysaccharides; the former, for example, occurs in the 1:3-β-glucan, laminarin (111), and xylose is present as a xylan in the red seaweed Rhodymenia palmata (112). D-galactose is the main structural unit in many sulphated algal polysaccharides while L-rhamnose has been reported in Ulva lactuca (56).

The polysaccharide material in Cladophora rupestris appears to be unique, however, in containing L-arabinose as a principal sugar component. The only other reported occurrence of this sugar in an algal polysaccharide is that from the blue-green weed, Anabaena cylindrica, (113), where it is present in one molar proportion with glucose, xylose, glucuronic acid, galactose and rhamnose (5:4:4:1:1 molar ratios).

The Heterogeneous Nature of the Polysaccharide Extract.

Although no fractionation of the polysaccharide extract with alcohol could be achieved, a glucan was ultimately isolated by chloroform extraction of its acetate. Furthermore, cetyl trimethyl ammonium bromide (114) when added to an aqueous solution of the extract formed an insoluble complex which gave, on hydrolysis and chromatography, galactose, arabinose, xylose and rhamnose in their usual proportions but was entirely devoid of glucose. This was found in the polysaccharide material remaining behind in the supernatant liquor and was accompanied by only trace quantities of the other four sugars (115). Electrophoresis experiments on the original purified material gave a Schlieren diagram with a symmetrical peak indicating a monodispersed polysaccharide, and it would seem that the detection of relatively small amounts of secondary carbohydrates is beyond the scope and sensitivity of the method. Attempts to precipitate a "κ - fraction" as in carrageenin (27, 28) were

unsuccessful while electroionophoresis experiments followed by treatment with toluidine blue, a reagent specific for sulphate groupings, gave a single discrete spot on an ionophoretogram. These experiments would seem to indicate that after removal of the glucan, the remaining carbohydrate material, henceforth referred to as "the polysaccharide", is monodispersed; indications of secondary components, unless present in small quantities, might reasonably be expected from one or more of the experiments described above. Until experiments prove the contrary, therefore, the material remaining after the removal of the glucan will be regarded as a single entity.

Investigation of the Glucan.

The method for the isolation of the glucan by chloroform extraction of the acetate was not discovered until an advanced stage in these investigations had been reached and in consequence all analytical work was carried out on the mixture of polysaccharide and glucan, henceforth referred to as "the polysaccharides".

The glucan, about 4% of the total polysaccharides (calculated on the proportion of glucose in the polysaccharides hydrolysate) could not be isolated completely free from the polysaccharide, chromatograms of the hydrolysate showing glucose together with trace amounts of galactose, arabinose and xylose. There was no evidence of rhamnose, but it should be pointed out that very small quantities of this sugar in a mixture are difficult to

detect chromatographically. The glucan appeared to be devoid of protein and contained less than 3% sulphate (probably derived from contaminating polysaccharide). It was essentially resistant to periodate attack since it was found in the oxypolysaccharide in relatively increased amount. Unless the structure is highly branched the predominant linkage must therefore be C_1 to C_3. It gave no colour with iodine and treatment of the polysaccharides with "laminarinase", an enzyme specific for β-1:3 - and β-1:4 - glucose linkages, caused the liberation of glucose, the only sugar evident on the chromatogram. Partial hydrolysis of the polysaccharides gave rise to two glucose oligosaccharides, one ($R_{Gal.}$ 0.81) was chromatographically and ionophoretically identical with laminaribiose, while the other moved at a rate ($R_{Gal.}$ 0.69) suggestive of a trisaccharide. This evidence points to the identity of the glucan as laminarin, possibly in a degraded form since its periodate consumption (1 mole periodate = 420 g. glucan) is higher than that recorded for the laminarin isolated from Laminaria cloustoni (1 mole periodate = 632 g. glucan) (111).

The Sulphur Content.

Sulphate was present in the polysaccharides to the extent of 19.6% and is undoubtedly etherally linked to the polysaccharide. The figure was not reduced on prolonged dialysis and, whereas ions such as calcium could be precipitated from solution, no sulphate could be detected until after hydrolysis. Furthermore, the sulphate content, 19.6%, and that calculated from the sulphated ash, 10.0%, agrees with the ratio 2:1 characteristic of polysaccharide ethereal sulphates.

$$2\ RO.SO_2.OM \rightarrow M_2SO_4 + SO_3 + \text{products of combustion.}$$

(R = sugar residue)
(M = cation)

As with other polysaccharides of this class, the sulphate was found to be extremely alkali stable. Treatment with N potassium hydroxide solution at 95° reduced the sulphate content to 15.1% in 4 hours and to 13.2% in 8 hours but was accompanied by extensive degradation of the carbohydrate material. This alkali stability of the sulphate is evident again in the methylated polysaccharide as this material still contains 16.2% sulphate after four methylations with alkali and methyl sulphate.

Assuming the polysaccharide molecule possesses a regular repeating unit, this, according to the molar proportions found, would consist in its simplest form of 8 arabinose (8A), 6 galactose (6G), 2 xylose (2X) and 1 rhamnose (1R) units.

Allowing 8% for protein and 4% for (unsulphated) glucan, the sulphur content corresponds to seven SO_3 Ca/2 groups to each of these repeating units.

As far as the author is aware, very little, if any, work has been done on pentose sulphates and it is impossible at this stage to assign the sulphate group to any particular carbon atom of the pentose sugar molecules. The work of Percival (53) on hexose sulphate would however indicate, from the alkali stability of these groups, that the galactose units carry the sulphate on C_4.

Reactions of the Polysaccharide with Sodium Metaperiodate.

It was found that 1 mole of periodate was consumed by 347 g. of the polysaccharides (36 hours' treatment). Measurements of formic acid release were indefinite since, due no doubt to over-oxidation effects, a steady state was never attained. Oxidation of the polysaccharides for 48 hours in sodium metaperiodate solution gave the oxypolysaccharide in high yields (80 - 87%) either by alcohol precipitation or freeze-drying of the dialysed solution. Hydrolysis of this oxypolysaccharide and quantitative estimation of the sugars showed the almost entire elimination of xylose and the removal of about two-thirds of the galactose, showing that these sugars must contain two contiguous unsubstituted hydroxyl groups. Arabinose, glucose and rhamnose, however, showed a relative increase in quantity, indicating that these sugars are

substantially immune from periodate attack.

In terms of the simple basic repeating unit already suggested (i.e. 8A: 6G: 2X: 1R) this would require the oxidation of all the xylose and four out of six of the galactose units. The percentage ratio of $\frac{\text{galactose}}{\text{arabinose}}$ theoretically required from the oxypolysaccharide hydrolysate would then be 0.30 whereas the ratio actually obtained was 0.27.

Partial Hydrolysis of the Polysaccharide.

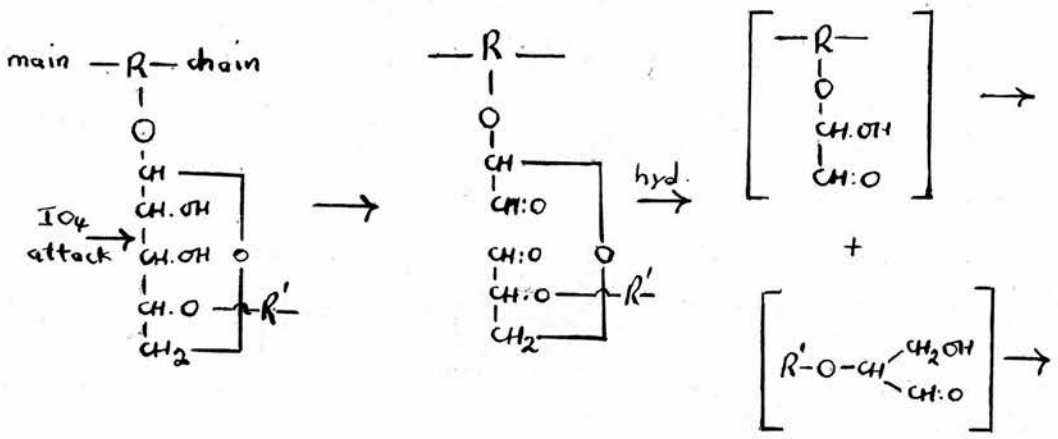
Mild oxalic acid hydrolysis experiments on the polysaccharides were found to conform to a simple pattern. Under the mildest conditions used, only galactose was liberated, indicating the presence of some galactofuranose residues in the molecule. When the temperature was raised to 50° both galactose and xylose were liberated together with a trace of arabinose, but as the hydrolysis proceeded the quantity of arabinose increased. The same sequence was observed on treating the polysaccharides with more concentrated oxalic acid solution at 95°. Galactose appeared after half an hour followed by xylose and arabinose after 2 hours. Glucose and rhamnose on the other hand were not liberated until hydrolysis had proceeded for 9 hours. Seliwanoff tests carried out on samples of the hydrolysis mixture at regular intervals gave positive results of varying intensity but it is difficult to know what significance, beyond degradative effects, can be placed on these observations.

Mild Acid Hydrolyses of the Polysaccharides and Oxypolysaccharide.

Comparison of the behaviour of the polysaccharides and oxypolysaccharide under the same mild hydrolysis conditions (0.1 N sulphuric acid for 3 hours at 100°) is of considerable interest. Whereas, in the former case, xylose and galactose are produced with only a trace of arabinose, the oxypolysaccharide showed the liberation of arabinose accompanied by only trace quantities of galactose and xylose. These results, in combination with those recorded in the previous section, indicate that galactose and xylose form the terminal units of the chains of the polysaccharide and their destruction by periodate exposes adjacent arabinose units directly to hydrolysis.

Repetition of this work on a larger scale gave comparable though not identical results. Separation of the hydrolysed portion from the hydrolytically resistant oxypolysaccharide material (Y) gave the former as a syrup which produced only a continuous broad streak on the chromatogram. On further hydrolysis with acid, however, discrete spots corresponding to galactose, arabinose and rhamnose were obtained. A possible explanation for this could be that the remnants of sugar residues left after attack by periodate are disintegrated by mild hydrolysis and fragments of these residues are left adhering to the main chain and part to the individual sugar units which are hydrolysed off, according to the manner

shown in the diagram.



Further treatment of the syrup with acid would therefore be necessary before complete liberation of the monosaccharide units from these extraneous chain fragments could be achieved.

The resistant material (Y) produced by mild hydrolysis of the oxypolysaccharide gave on complete hydrolysis arabinose together with lesser amounts of galactose and glucose, the last named sugar arising, of course, from the glucan. This material (Y) contained 20% sulphate and gave a single spot on the electrionophoretogram sprayed with toluidine blue, a reagent specific for sulphate groups. The glucan and any other non-sulphated polysaccharides would not be detected by these means. As the material (Y) was recovered from a solution which had been dialysed in a cellophane bag against running water, both the mildly hydrolysed oxypolysaccharide and the glucan must consist of fairly large molecules.

From these results, it seems probable that this resistant material (Y: 13% of starting material) does in fact constitute the backbone of the molecule and that this consists mainly of arabinose together with some galactose residues either or both of which carry sulphate groups.

Expressed in terms of the simplest possible repeating unit (8A: 6G: 2X: 1R), approximately four galactose units in six are oxidised by periodate leaving two galactose units to be incorporated in the main chain. Furthermore, the fact that some arabinose is also liberated on mild hydrolysis of the oxypolysaccharide indicates that one or more of these units are probably present in the side chain. It has also been observed that increasingly vigorous hydrolysis of the oxypolysaccharide yields a resistant material (Z) with a higher $\frac{G}{A}$ ratio, a result which suggests the progressive removal of side-chain arabinose as hydrolysis becomes more severe. That this liberated arabinose is derived from the main chain seems less likely since this would entail a considerable overall reduction in the size of the molecule, possibly reducing it to dialysable dimensions.

It is difficult to derive much information concerning the origin of the rhamnose. It appears itself to be highly resistant to the action of periodate and to all but the more severe hydrolytic conditions. In the oxypolysaccharide, however, it is readily liberated during mild hydrolysis which suggests that rhamnose might be shielded in the polysaccharide by residues susceptible themselves to periodate attack.

Separation and Hydrolysis of the Oligosaccharides.

The oligosaccharides, produced by mild oxalic acid hydrolysis of the polysaccharides, were separated on a charcoal-celite column and purified by paper chromatography. Each was hydrolysed and, since the quantities of hydrolysate syrups obtained were insufficient for quantitative analysis, the ratios of the sugars produced in each case were estimated visually. In order to ensure that these oligosaccharides are not artefacts, a synthetic mixture was subjected to an identical hydrolysis procedure (a small quantity of sulphuric acid being added comparable to that liberated from the ethereal sulphate groups during the authentic hydrolysis). Charcoal-celite separation was carried out as for the authentic syrup but no trace of oligosaccharides could be found.

The most striking feature of these partial hydrolysis experiments of the polysaccharides was the emergence of a series of oligosaccharides consisting solely of arabinose which, according to their mobility on the chromatogram, must in the case of the slowest moving member of the series ($R_{Gal.}$ 0.28) be composed of at least three if not four arabinose units. The second arabinose oligosaccharide ($R_{Gal.}$ 0.58) is probably a trisaccharide while the remaining two members ($R_{Gal.}$ 0.87 and 0.95 from cellulose column) were presumably disaccharides since they both moved at comparable though not identical rates to 3-O-β-L-arabopyranosyl-L-arabinose, $R_{Gal.}$ 0.78.

These results are in accordance with the view that the backbone of the polysaccharide is essentially arabinose in character while the emergence of two disaccharides demonstrates the existence of two sets of A - A linkages within the molecule; possibly one may arise from a main chain linkage, while the other is the side chain linkage, thus:

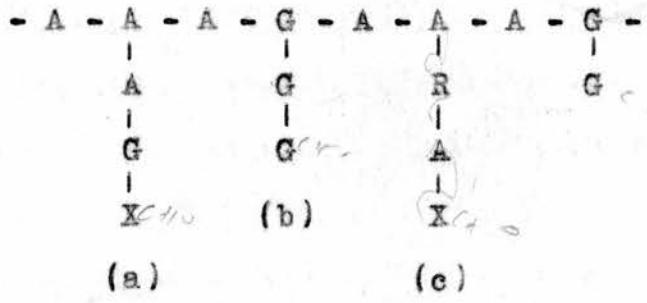
```
- A - A - main chain
    |
    A
```

The other oligosaccharides isolated were as follows:

		R_{Gal}.	
(1)	A;G;X (2;1;1)	0.38	
(2)	A;G;X (1;1:1)	0.58	
(3)	A;X (1:1)	0.68	
(4)	G	0.29	
(5)	Gl	(0.69 (0.81	(see section on glucan).

Assuming that the molecule possesses a simple repeating unit of the type already suggested it is possible, on the basis of this and earlier work, to suggest a tentative formula for the general disposition of the sugar residues

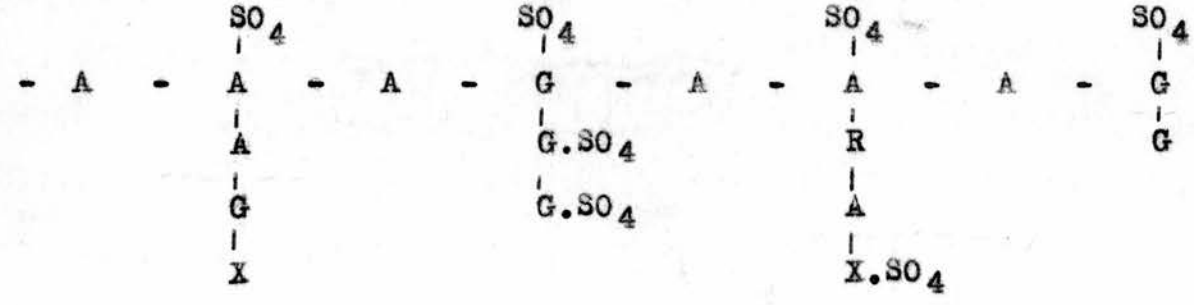
within that unit:



It is not, of course, suggested that this is in any sense a unique solution, but only that it seems to provide one of the simplest interpretations of the available evidence.

Oligosaccharides (1) and (2) could be derived from side-chain (a), (3) from side-chain (c), while (4) could arise from side-chain (b). At the same time, this formula would give rise to two possible arabinose disaccharides and a series of higher arabinose oligosaccharides.

As has already been mentioned in an earlier section, the sulphate content of this polysaccharide allots seven SO_3 Ca/2 groups to each repeating unit. Some of these groups undoubtedly reside on the backbone; a possible pattern for these, in keeping with the evidence from methylation studies, would be:



Mild hydrolysis would be expected to release galactose and xylose first, followed by arabinose. Following the periodate

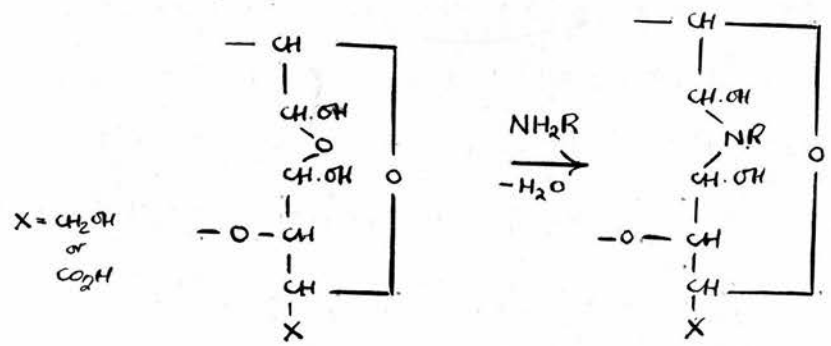
oxidation of the side-chain galactose and xylose units, two out of the eight arabinose units would then be directly exposed to hydrolytic agents, which is in keeping with the experimental observations.

Hypoiodite Oxidation and the Average Molecular Size of the Polysaccharide.

Reaction with sodium hypoiodite showed one oxidisable group in 31,200 g. of polysaccharide which indicates that the polysaccharide has a large molecular weight. The reducing power of the polysaccharide is, however, too small to allow precise measurement and so the results cannot be used to calculate an accurate molecular weight.

Barry Degradation Experiments.

According to Barry (88), polysaccharide sugar residues opened out by periodate oxidation are capable of forming complexes with compounds such as phenylhydrazine and isoniazid (isonicotinoylhydrazine) (RNH_2) and he considers that the following reaction occurs:



The action of phenylhydrazine acetate and of isoniazid on the oxypolysaccharide gave poor yields of the complexes and these possessed varying nitrogen contents. Attempts to isolate crystalline derivatives or to recover the oxypolysaccharide were unsuccessful and it appears that extensive degradation of the carbohydrate material occurs. Owing to the poor yields obtained, the failure to isolate crystalline derivatives and to the fact that further investigations along these lines seemed unlikely to yield more information concerning the structure of the polysaccharide, these experiments were abandoned.

Methylation Studies on the Polysaccharide.

Different methods of acetylation were investigated and a product with an acetyl content of 20.6% was finally obtained. Simultaneous deacetylation and methylation by the method of Haworth in the cold in an atmosphere of nitrogen gave a product containing 19.6% methoxyl. The methylation was repeated three times at 45° and a product with 25% methoxyl isolated.

Attempts to raise the methoxyl content by Purdie methylations, by the use of Purdie reagent and dimethyl formamide, and by the use of thallous hydroxide and methyl iodide were unsuccessful.

The main bulk of the polysaccharide was acetylated three times and the acetylated material deacetylated and methylated by the method of Haworth. Considerable losses of material occurred during this methylation (42% recovery) but it is difficult to know to what extent these may be attributed to mechanical losses due to faulty cellophane tubing during the removal of inorganic ions by dialysis.

The methoxyl content required for a polysaccharide built up from the suggested repeating unit would be 30%. However, the methoxyl content could not be raised above 25.1% which may well be due to the shielding effect of the sulphate residues preventing complete methylation of all the free hydroxyl groups.

The various methylated derivatives obtained after methanolysis and hydrolysis of the methylated material are detailed below. The weights quoted are in excess of those recorded in the experimental section, allowance having been made for the amounts of each sugar present in the overlap fractions. It should be added that fractions varied considerably in the amount of wax contamination and, since losses in purification were considerable, particularly in the higher methylated fractions, the weights quoted for them must be regarded as tentative.

While it is realised that the isolation of an authentic crystalline derivative is necessary for a complete characterisation of a methylated sugar, the complex mixture of methylated derivatives in this particular material has made this impossible for fractions which were isolated in very small quantities. Identity in these instances has been tentatively assumed from demethylation, chromatography and rotational data. However, in those cases where sufficient amounts of material have been isolated, experiments are in progress for the preparation of crystalline compounds.

Methylation Studies.

Arabinose.	R_G.	Weights (mg.).
2:3:5-Tri-O-methyl	0.94	Trace
Di-O-methyl (possibly 2:3)	0.63	117
2:4-Di-O-methyl	0.54	119
2-O-methyl	0.38	610
3-O-methyl	0.28	266
Free arabinose	0.14	310
	Total:	1.422.g.

The proportion of arabinose derivatives, 49% of the total weight of syrup recovered, is a little higher than might be expected from the amount of free sugar present in the polysaccharide hydrolysate, i.e. 43% (allowance made for glucan removal). However, if any degradation of the molecule occurred during methylation the arabinose occupying a central position would gain a measure of protection and this result might therefore be anticipated.

The trace of 2:3:5-tri-O-methyl arabinose, which was identified tentatively by chromatographic analysis, could only originate from a terminal arabofuranose unit. The di-O-methyl arabinose, which gave arabinose as the only free sugar on demethylation, was tentatively identified as the 2:4-di-O-methyl derivative from its chromatographic mobility and by its specific rotation and by the rotation of its derived lactone. The

presence of this derivative indicates that a small amount of the arabinose occurs in the pyranose form with 1:3 linkages. The 2-O-methyl arabinose and the 3-O-methyl arabinose were demethylated to give arabinose and identified by their chromatographic and electrophoretic mobilities in comparison with authentic specimens, by their rotations and by the rotations of the derived lactones.

Free arabinose, isolated in a crystalline state, together with the monomethyl derivatives, probably originate from main chain units some of which are probably protected from methylation by the presence of sulphate residues.

Except for the trace of tri-O-methyl sugar and the small quantity of the possible 2:3-di-O-methyl derivative isolated, these results are in keeping with the earlier work that the arabinose is largely unattacked by periodate and does constitute the central features of the polysaccharide molecule.

Galactose.	R_G	Weight (mg.).
2;3;4:6-Tetra-O-methyl	0.88	37
2:3:5-Tri-O-methyl	0.86	138
Tri-O-methyl (probably 2:3:4)	0.63	117
2:4-Di-O-methyl	0.38	85
2-O-methyl	0.21	270
Free galactose	0.08	508
	Total:	1.155 g.

The amount of galactose containing syrup to total recovered, 40%, is of the order expected from the amount of free sugar present in the polysaccharide hydrolysate (39%). However, the proportion (approximately 25%) of the total galactose open to periodate attack as indicated by the above derivatives is smaller than the quantitative estimations on the oxypolysaccharide hydrolysate suggest. This may be due partly to undermethylation of the polysaccharide and partly to losses incurred in the recovery and purification of the higher methylated fractions from the hydrolysis syrup.

The tetra-O-methyl galactose failed to crystallise; it was demethylated to galactose and tentatively identified by methoxyl content, chromatographic mobility and rotation. The 2:3:5-tri-O-methyl galactose, the 2:4-di-O-methyl galactose and the 2-O-methyl galactose all gave galactose on demethylation and had identical chromatographic mobilities to those of authentic derivatives. The first and last of these were further characterised by the rotation of their derived lactones and the 2:4-di-O-methyl derivative crystallised with the correct melting point and showed no depression on admixture with an authentic specimen. The 2:3:4-tri-O-methyl galactose was obtained in admixture with a dimethyl arabinose derivative and was tentatively identified from the facts that it gave galactose on demethylation, that its rate of movement on the paper chromatogram was identical with an authentic sample of 2:3:4-tri-O-methyl galactose and that its calculated rotation

from the rotation of the mixture is in good agreement with the recorded figure for this derivative.

The methylated galactose derivatives isolated are in keeping with the earlier experimental results. The isolation of the tetramethyl galactose shows that some of the galactose functions as end units while the 2:3:5-tri-O-methyl derivative indicates the presence of galactofuranose residues, a result in keeping with the observation that 0.1 N oxalic acid liberates galactose at room temperature. The other tri-O-methyl sugar, if indeed it is the 2:3:4 derivative, originates from a residue susceptible to periodate attack and must therefore be present in a side-chain. The monomethyl derivative and the free galactose probably arise from galactose residues situated in the main chain and at branch points, as such residues, if they carried sulphate groups, would have at the most one hydroxyl group free for methylation.

Xylose.	R_G.	Weight (mg.).
2:3:4-Tri-O-methyl	0.94	70
2:3-Di-O-methyl	0.74	58
	Total:	128

The total xylose recovery, < 5%, is lower than expected, but this may again be due to losses in recovery and purification.

The 2:3:4-tri-O-methyl derivative was tentatively identified by chromatographic mobility in several solvents in comparison

with an authentic specimen and by rotation. Chromatography, ionophoresis and rotational data of the dimethyl derivative compared with an authentic specimen characterised this as 2:3-di-O-methyl xylose.

The isolation of the trimethyl derivative confirms the earlier evidence that part at least of the xylose functions as end units. The 2:3-dimethyl derivative may arise from 1:4 - linked inner members of the branch chains or from end units which carry a sulphate residue on C_4. The possibility that it originates from undermethylation is less likely as no other di-O-methyl xylose derivative was isolated and it seems improbable that such an effect would be entirely localised to carbon 4 of the xylose residue.

Rhamnose.	R_G.	Weight (mg.).
Di-O-methyl (probably 2:4- and 3:4-)	0.88	34
4-O-methyl	0.57	78
	Total:	112

The percentages of rhamnose derivatives (4%) to the total recovered is of the order expected from estimations of this sugar in the polysaccharides hydrolysate. The derivatives, all of which gave rhamnose on demethylation, have been identified tentatively by chromatography and ionophoresis in comparison with authentic specimens and by their specific rotations. If correct, they show that this sugar is joined in the main by

a C_1 to C_3 linkage; such units would be immune to periodate attack.

Apart from the presence of the relatively small quantity of the 3:4 - derivative, the isolation of the other derivatives support the earlier findings that this residue is essentially immune from periodate attack and therefore cannot contain any α-glycol groupings.

Sulphated polysaccharides are difficult to fully methylate (116) and it seems probable that some of the hydroxyl groups of the monomethyl and free sugars isolated from the hydrolysate of the methylated polysaccharide escaped methylation because of the shielding effect of the sulphate groups. Furthermore, the polysaccharide is bound up with protein or peptide material and this too may hinder complete methylation of the sugar residues. Methylation studies do, however, support the general picture of the structure proposed from the results of the earlier experiments inasmuch as they show that the polysaccharide contains a complex molecule built up from a backbone consisting of arabinose and galactose residues in the ratio of 3:1. The molecule is branched, the inner members of the branches consisting of arabinose, rhamnose and galactose residues, while the latter sugar and xylose are present as terminal units.

The polysaccharide is only slightly reducing and therefore C_1 is involved in the linkages between adjacent units but conclusive evidence as to which of the other carbon atoms are involved in the linkage has not been obtained in every case, although the presence of some 1:3 linked galactose, arabinose and rhamnose and some 1:4 linked xylose has been established. In spite of the positive rotation of the polysaccharide and of its methylated derivative, no conclusion can be drawn as to the α or β nature of the linkages.

SUMMARY.

1. Water-soluble polysaccharides have been isolated from the green seaweed, Cladophora rupestris.

2. The crude extracts had a nitrogen content of 2 - 4% and this has been shown to be due to protein contamination.

3. Standard methods for protein removal did not reduce the nitrogen content below 1% and it is suggested that the residual nitrogenous material is chemically bound to the polysaccharide.

4. The purified material had $[\alpha]_D^{20°}$ + 69° (c., 1.0 in water); nitrogen content 1.26%; ash (as sulphate) 16.1% consisting of calcium 22.1%, iron 4.4%, sodium 3.7%, potassium 0.4%, oxides of aluminium and silicon 6.1%, sulphate 62.0%; total sulphate 19.6% cf. sulphate calc. from ash 10.0%; methoxyl and acetyl content, nil.

5. The mixture of polysaccharides was acetylated (CH_3CO, 20.3%) and chloroform extraction of this acetylated material separated a glucan. This constituted about 4% of the mixture and from the results of different experiments is concluded to be unsulphated laminarin.

6. Standard separation procedures carried out on the remaining polysaccharide material gave no indication of further heterogeneity and hydrolysis revealed the presence of D-galactose, L-arabinose, D-xylose and L-rhamnose in the approximate molar proportions 6:8:2:1 respectively. All four sugars were separated in crystalline form and characterised by the preparation of authentic crystalline derivatives.

Ketose, uronic acids and amino-sugars were shown to be absent.

7. The sulphate groups were resistant to alkaline hydrolysis; a small reduction in sulphate content did take place on treatment with hot alkali, but this was accompanied by extensive degradation of the carbohydrate material.

8. Partial hydrolysis with acid preferentially removed galactose and xylose from the polysaccharide, indicating that these units are situated at the ends of the polysaccharide chains.

9. Periodate uptake studies showed that one molecule of periodate was equivalent to 347 g. of polysaccharide. Formic acid release measurements were inconclusive, acid being released continually.

10. Periodate oxidation of the purified extract led to the elimination of virtually all the xylose and about two-thirds of the galactose, showing that these residues contain two contiguous unsubstituted hydroxyl groups and again indicating that these two sugars occur as end units or in branch chains of the polysaccharide molecule. The residual material, the oxypolysaccharide, was isolated in 80 - 87% yield.

11. Mild hydrolysis of the oxypolysaccharide removed galactose, rhamnose and arabinose and left a sulphated, non-dialysable material containing arabinose together with some galactose. These results suggest that the main back bone of the polysaccharide molecule is made up of arabinose and galactose and that arabinose and rhamnose occur in the branch chains with the galactose and xylose.

12. The oligosaccharides produced by hydrolysis of the polysaccharide with aqueous oxalic acid have been separated and investigated. A tentative formula for a simple repeating unit, containing 8 arabinose, 6 galactose, 2 xylose and 1 rhamnose residues and carrying 7 sulphate groups, has been proposed. This formula incorporates the different oligosaccharides isolated and is in harmony with the earlier results.

13. Hypoiodite uptake measurements were very small and serve only to indicate that the polysaccharide has a high molecular weight.

14. Different methods of methylation were carried out but the methoxyl content of the methylated polysaccharide could not be raised above 25.10%.

15. Hydrolysis of the methylated material and separation by cellulose column chromatography of the mixture of partly methylated sugars produced led to the isolation and tentative identification of tetra-O-methylgalactopyranose, the tri-O-methyl derivatives of galactose, xylose and arabinose, the di-O-methyl derivatives of galactose, xylose, arabinose and rhamnose, the mono-O-methyl derivatives of galactose, arabinose and rhamnose together with free galactose and arabinose. The principal fractions isolated and characterised were: 2:3:5-tri-O-methyl galactose, 2:3-di-O-methyl xylose, 2-O-methyl galactose, 2-O-methyl and 3-O-methyl arabinose, 4-O-methyl rhamnose, free galactose and arabinose. The isolation of these derivatives is in agreement with the proposed repeating unit.

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